TO ALL WHOM IT MAY CONCERN:

Be it known that we, Gyanendra Kumar, a citizen of the United States of America residing at 115 Brookridge Lane, Guilford, Connecticut 06437, Patricio Abarzua, a citizen of Chile residing at 17 Woodside Avenue, West Caldwell, New Jersey 07006, and Michael Egholm, a citizen of Denmark residing at 8 Dogwood Court, Woodbridge, Connecticut 06525, have invented new and useful improvements in

DETECTION METHOD USING DISSOCIATED ROLLING CIRCLE AMPLIFICATION

for which the following is a specification.

DETECTION METHOD USING DISSOCIATED ROLLING CIRCLE AMPLIFICATION

FIELD OF THE INVENTION

The disclosed invention is generally in the area of detection of analytes, and specifically in the area of detection of analytes using rolling circle amplification.

BACKGROUND OF THE INVENTION

The information content of the genome is carried as deoxyribonucleic acid (DNA). The size and composition of a given genomic sequence determines the form and function of the resultant organism. In general, genomic complexity is proportional to the complexity of the organism. Relatively simple organisms such as bacteria have genomes of about 1-5 million megabases while mammalian genomes are approximately 3000 megabases. The genome is generally divided into distinct segments known as chromosomes. The bacterium *Escherichia coli* (*E. coli*) contains a single circular chromosome, whereas the human genome consists of 24 chromosomes.

Genomic DNA exists as a double-stranded polymer containing four DNA bases (A, G, C, and T) tethered to a sugar-phosphate backbone. The order of the bases along the DNA is the primary sequence of the DNA. The genome of an organism contains both protein coding and non-coding regions, including exons and introns, promoter and gene regulatory regions, and non-functional DNA. Genome analysis can provide a quantitative measure of gene copy number and chromosome number, as well as the presence of single base differences in the primary sequence of the DNA. Single base changes that are inherited are referred to as polymorphisms, whereas those that are acquired during the life of an organism are known as mutations. Genomic analysis at the DNA level does not provide a measure of gene expression (that is, the process by which RNA and protein copies of the coding sequences are synthesized).

All of the cells from a given organism are assumed to contain identical genomes, while genomes from different individuals of the same species are typically about 99.9% identical. The 0.1% polymorphism rate among individuals (Wang *et al., Science* **280**: 1077 (1998)) is significant in that approximately three million polymorphisms are expected to be found upon complete sequencing of any two human genomes. If single base changes occur in protein coding segments, polymorphisms can alter the protein sequence and therefore change the biochemical activity of the protein.

The DNA genome consists of discrete functional regions known as genes. Genomes of simple organisms such as bacteria contain approximately 1000 genes (Fleischmann et al., *Science* 269: 496 (1995)), whereas the human genome is estimated to contain about 100,000 genes (Fields et al., *Nature Genet.* 7: 345 (1994)). Genomic analysis at the mRNA level can be used as a measure of gene expression. Expression levels for each gene are determined by a combination of genetic and environmental factors. The genetic factors include the precise DNA sequence of gene regulatory regions such as promoters, enhancers, and splice sites. Polymorphisms in the DNA are thus expected to contribute some of the differences in gene expression among individuals of the same species. Expression levels are also affected by environmental factors, including temperature, stress, light, and signals that lead to changes in the levels of hormones and other signaling substances. For this reason, RNA analysis provides information not only about the genetic potential of an organism, but also about changes in functional state (M. Schena and R.W. Davis, *DNA Microarrays: A Practical Approach.* (Oxford University Press, New York, 1999) 1-16.)

The second step in gene expression is the synthesis of protein from mRNA. A unique protein is encoded by each mRNA, such that every three nucleotides of mRNA encodes one amino acid of the polypeptide chain, with the linear order of the nucleotides represented as a linear sequence of amino acids. Once synthesized, the protein assumes a unique three-dimensional conformation that is determined largely by the primary amino acid sequence. Proteins impart the functional instructions of the genome by performing a wide range of biochemical activities including roles in gene regulation, metabolism, cell structure, and DNA replication.

Individuals in a population may have differences in protein activity due to polymorphisms that either alter the primary amino acid sequence of the proteins or perturb steady state protein levels by altering gene expression. Similar to mRNA levels, protein levels can also change in response to changes in the environment; moreover, protein levels are also subject to translational and post-translational control which do not effect mRNA levels directly (Schena and David, 1999). Proteomics analysis provides data on when or if a predicted gene product is actually translated, the level and type of post-translational modification it may undergo and its relative concentration compared with other proteins (Humphrey-Smith and Blackstock, *J.*

Protein. Chem. 16: 537-544 (1997)). After DNA is transcribed into mRNA, the exons may be spliced in different ways before being translated into proteins. Following the translation of mRNA by ribosomes, proteins are usually post-translationally modified by the addition of different chemical groups such as carbohydrate, lipid and phosphate groups, as well as through the proteolytic cleavage of specific peptide bonds. These chemical modifications are crucial to modulating protein function but are not directly coded for by genes. Furthermore, both mRNA and protein are continually being synthesized and degraded, and thus final levels of protein are not easily obtainable by measuring mRNA levels (Patton, *J. Chromatogr.* 722: 203-223, (1999); Patton et al., *J. Biol. Chem.* 270: 21404-21410 (1995)). So while mRNA levels are often extrapolated to indicate the levels of expressed proteins, it is not surprising that there is little correlation between the abundance of mRNA species and the actual amounts of proteins that they code for (Anderson and Seilhamer, *Electrophoresis* 18: 533-537; Gygi et al., *Mol. Cell. Biol.* 19: 1720-1730 (1999)).

A growing body of evidence suggests that changes in gene and protein expression may correlate with the onset of a given human disease (Schena and Davis, 1999). Proteomic analysis of disease tissues should allow the identification of proteins whose expression is altered in a given illness. Many small molecules may also alter protein expression at a global level. Combining information about altered expression in a disease state with the changes that result from treatment with a small molecule would provide valuable information about classes of molecules that may be effective in combating a given disease. Proteomics thus has a role in processes such as lead compound screening and optimization, toxicity, pharmacodynamics, and drug efficacy.

A pivotal component of proteomics is its ability to accurately quantify vast numbers of proteins accurately and reproducibly. Typically, proteomics entails the simultaneous separation of proteins from a biological sample, and the quantitation of the relative abundance of the proteins resolved during the separation. Proteomics currently relies heavily on two-dimensional (2-D) gel electrophoresis. However, obtaining information concerning global protein expression using 2-D gels is technically difficult, and semiautomated procedures to carry out this process are in their infancy (Patton, *Biotechniques* 28: 944-957 (2000)). Furthermore, the commonly used stains for evaluating protein expression in 2-D gels (such as Coomassie Blue, colloidal

gold and silver stain) do not provide the requisite dynamic range to be effective in this capacity. These stains are linear over only a 10- to 40-fold range, whereas the abundance of individual proteins differs by as much as four orders of magnitude (Brush, *The Scientist* 12:16-22, 1998; Wirth and Romano, *J. Chromatogr* 698: 123-143 (1995)). In addition, low abundance proteins, such as transcription factors and kinases that are present in 1-2000 copies per cell, often represent species that perform important regulatory functions. The accurate detection of such low-abundance proteins is an important challenge to proteomics. Methods have recently been introduced to directly quantify the relative abundance of proteins in two different samples by mass spectrometry. However, the linear dynamic range of these methods has been demonstrated over only a four- to ten- fold range (Gygi et al. 1999; Oda et al., *Proc. Natl. Acad. Sci USA* 96: 6591-6596 (1999)).

It has been noted that developing microarray technologies would make possible the simultaneous, ultra-sensitive measurement of hundreds or even thousands of substances in a small sample (Ekins, *Clin. Chem.* 44: 2015-2030 (1998)). This approach has been difficult to put into practice, however, because the extremely small volumes (about 0.5-5 nl) of sample used to create spots on these microarrays makes it necessary to utilize methods of analyte detection that are extremely sensitive. Rolling Circle Amplification (RCA) driven by DNA polymerase can replicate circular oligonucleotide probes with either linear or geometric kinetics under isothermal conditions (Lizardi et al., *Nature Genet.* 19: 225-232 (1998)). If a single primer is used, RCA generates in a few minutes a linear chain of hundreds or thousands of tandemly-linked DNA copies of a target which is covalently linked to that target. Generation of a linear amplification product permits both spatial resolution and accurate quantitation of a target. DNA generated by RCA can be labeled with fluorescent oligonucleotide tags that hybridize at multiple sites in the tandem DNA sequences. RCA can be used with fluorophore combinations designed for multiparametric color coding (Speicher et al., *Nature Genet.* 12:368-375 (1996)), thereby markedly increasing the number of targets that can be analyzed simultaneously. RCA technologies can be used in solution, *in situ* and in microarrays. In solid phase formats, detection and quantitation can be achieved at the level of single molecules (Lizardi et al., 1998).

BRIEF SUMMARY OF THE INVENTION

Disclosed are compositions and methods for detecting small quantities of analytes such as proteins and peptides. The method involves associating a DNA circle with the analyte and subsequent release and rolling circle replication of the circular DNA molecule. Thus, the disclosed method produces an amplified signal, via rolling circle amplification, from any analyte of interest. The amplification is isothermic and can result in the production of a large amount of nucleic acid from each primer.

The disclosed method is preferably used to detect and analyze proteins and peptides. In some embodiments, multiple proteins can be analyzed using solid supports, such as microtiter dishes, with which multiple different proteins or analytes are directly or indirectly associated (if they are present in the sample being tested). An amplification target circle is then associated with the various proteins using a conjugate of the circle and a specific binding molecule, such as an antibody, that is specific for the protein to be detected. Amplification target circles not associated with the proteins are removed, the amplification target circles that are associated with the proteins are decoupled from the specific binding molecule and replicated. Rolling circle replication primed by rolling circle replication primers results in production of a large amount of DNA. Use of exponential rolling circle amplification (ERCA), where the strand replicated from the amplification target circle is replicated using a second primer and both replicated strands generate further replicated strands, is preferred. Amplification products can be detected in real time using, for example, Amplifluor™ primers. The amplified DNA serves as a readily detectable signal for the proteins. Different proteins can be distinguished in several ways. For example, each different protein can be associated with a different amplification target circle which in turn is replicated to produce amplified DNA. The result is distinctive amplified DNA for each different protein. The different amplified DNAs can be distinguished using any suitable sequence-based nucleic acid detection technique. In this form of the method, many proteins can be detected in the same amplification reaction. Alternatively, the location of the amplified DNA on a solid support can indicate the protein involved if different proteins are immobilized at pre-determined locations on the support.

Another embodiment of the disclosed method involves comparison of the proteins expressed in two or more different samples. The information generated is

analogous to the type of information gathered in nucleic acid expression profiles. The disclosed method allows sensitive and accurate detection and quantitation of proteins expressed in any cell or tissue. The disclosed method also allows the same analyte(s) from different samples to be detected simultaneously in the same assay.

It is an object of the present invention to provide a method for detecting small quantities and concentrations of analytes.

It is a further object of the present invention to provide a method for detecting small quantities and concentrations of multiple analytes in samples.

It is a further object of the present invention to provide a method for amplifying the signal of an analyte to be detected.

It is a further object of the present invention to provide an automated method for detecting small quantities and concentrations of multiple analytes in samples.

It is a further object of the present invention to provide a method for profiling the presence of multiple analytes in a sample.

It is a further object of the present invention to provide a method for comparing profiles of the presence of multiple analytes in different samples.

It is a further object of the present invention to provide a method for assessing the interaction of compounds with molecules of interest.

It is a further object of the present invention to provide a method for detecting small quantities and concentrations of proteins and peptides.

It is a further object of the present invention to provide a method for detecting small quantities and concentrations of multiple proteins and peptides in samples.

It is a further object of the present invention to provide a method for amplifying the signal of a protein or peptide to be detected.

It is a further object of the present invention to provide an automated method for detecting small quantities and concentrations of multiple proteins and peptides in samples.

It is a further object of the present invention to provide a method for profiling the presence of multiple proteins and peptides in a sample.

It is a further object of the present invention to provide a method for comparing profiles of the presence of multiple proteins and peptides in different samples.

It is a further object of the present invention to provide a method for assessing the interaction of compounds with proteins and peptides of interest.

It is a further object of the present invention to provide compositions for detecting small quantities and concentrations of analytes.

It is a further object of the present invention to provide compositions for detecting small quantities and concentrations of proteins and peptides.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are diagrams of examples of two forms of the disclosed method. In Figure 1A, a reporter binding molecule (anti-human IgG with circle) is associated with a protein (HIV P24 protein) via an anti-HIV P24 antibody. The protein that is attached to Micro Amp tubes. The specific binding molecule of the reporter binding molecule is an anti-human IgG. In Figure 1B, a reporter binding molecule (anti-biotin antibody with circle) is associated with a protein (HIV P24 protein) that is associated with an anti-HIV P24 antibody. The anti-HIV P24 antibodies are attached to Micro Amp tubes, thus associating the protein with the Micro Amp tubes. The specific binding molecule of the reporter binding molecule is an anti-biotin antibody. The amplification target circle of the reporter binding molecule is associated with the specific binding molecule via a circle capture probe.

Figure 2 is a graph of antibody (micrograms in 30 µl) versus absorbance at 450 nm. This shows the amount of coating by the antibody when different amounts of antibody are used.

Figure 3 is a diagram of a comparison of association of reporter binding molecules to cognate and non-cognate analytes. The "analytes" are anti-biotin antibodies (cognate) and mouse IgG (non-cognate). The non-cognate analyte serves as a control. The reporter binding molecules consists of biotin (the specific binding molecule), an oligonucleotide (the circle capture probe), and an 1822 circle (the amplification target circle) which is complementary to the oligonucleotide. The reporter binding molecule interacts only with the anti-biotin antibodies. Decoupled amplification target circles are amplified by ERCA using an Amplifluor™ primer (P1), a secondary DNA strand displacement primer (P2), and Bst DNA polymerase.

Figures 4A, 4B, and 4C are graphs of time (in "cycles," which are 2 minute time units) versus fluorescence. The difference in delta Ct when using different numbers of reporter binding agents is shown between the three graphs.

Figure 5 is a diagram of a comparison of association of partial reporter binding molecules to cognate and non-cognate analytes. The "analytes" are anti-biotin antibodies (cognate) and mouse IgG (non-cognate). The non-cognate analyte serves as a control. The partial reporter binding molecules consists of biotin (the specific binding molecule), and an oligonucleotide (the circle capture probe). The partial reporter binding molecule interacts only with the anti-biotin antibodies. The amplification target circles, which are complementary to the oligonucleotide, are annealed to the circle capture probe after the partial reporter binding molecule is associated with the analyte. Decoupled amplification target circles are amplified by ERCA using an Amplifluor™ primer (P1), a secondary DNA strand displacement primer (P2), and Bst DNA polymerase.

Figure 6 is a graph of the number of circle capture probes used (in thousands) versus the change in counts (in minutes).

Figure 7 is a diagram of an example of immunoRCA involving amplification target circles associated with specific binding molecules via base pairing to circle capture probes. Micro Amp tubes coated with anti-IL8 antibodies (analyte capture agents) are brought into contact with IL8 (analyte) and the IL8 binds to the antibodies. A biotinylated anti-IL8 antibody is brought into contact with the captured IL8 and they bind. Reporter binding molecules (comprising an anti-biotin antibody, a circle capture probe and an amplification target circle) are brought into contact with the biotinylated anti-IL8 antibody and they bind. This associates the reporter binding molecule with the analyte (IL8) indirectly (via the biotinylated anti-IL8 antibody). The amplification target circle is decoupled from the reporter binding molecule by disrupting the base pairing between the amplification target circle and the circle capture probe and amplified in ERCA.

Figure 8 is a graph of the amount of IL8 (in pg/ml) versus the change in counts (in minutes).

Figure 9 is a diagram of an example of immunoRCA involving amplification target circles coupled to specific binding molecules via circle linkers having cleavable

bonds. Anti-analyte antibodies (analyte capture agents) are brought into contact with analyte and the analyte binds to the antibodies. Biotinylated anti-analyte antibodies are brought into contact with the captured analyte and they bind. Reporter binding molecules (comprising an anti-biotin antibody, a circle linker containing a cleavable bond, and an amplification target circle) are brought into contact with the biotinylated anti-analyte antibody and they bind. This associates the reporter binding molecule with the analyte indirectly (via the biotinylated anti-analyte antibody). The amplification target circle is decoupled from the reporter binding molecule by cleaving the cleavable bond and the circle capture probe and amplified in ERCA.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed are compositions and methods for detecting small quantities of analytes such as proteins and peptides. The method applies the power of nucleic acid signal amplification to the detection of non-nucleic acid analytes. Detection of such analytes--for which there are no amplification techniques comparable to nucleic acid amplification techniques--has generally depended on detection of sufficient quantities of the analyte or the use of extremely sensitive labels. The use of such labels is both cumbersome and limited. The disclosed method provides a simple and sensitive way to produce an amplified signal for any desired analyte.

The disclosed method is a form of rolling circle amplification (RCA) where a reporter binding molecule provides the amplification target circle for amplification. The disclosed method allows RCA to produce an amplified signal (that is, tandem sequence DNA (TS-DNA)) based on association of the reporter binding molecule with a target molecule (also referred to as an analyte). The specific amplification target circle that is a part of the reporter binding molecule provides the link between the specific interaction of the reporter binding molecule to an analyte (via the affinity portion of the reporter binding molecule) and RCA. Once the reporter binding molecule is associated with an analyte, a rolling circle replication primer is hybridized to the amplification target circle (ATC) of the reporter binding molecule, followed by amplification of the ATC by RCA (a secondary DNA strand displacement primer is also used if exponential RCA is performed). The disclosed method can be performed using any analyte. Preferred analytes are proteins, peptides, nucleic acids, including amplified nucleic acids such as TS-DNA and amplification target circles, antigens and

ligands. Target molecules for the disclosed method are generally referred to herein as analytes.

The amplification target circle is released from the reporter binding molecule prior to or during amplification. Such release, referred to herein as decoupling, can be accomplished in any suitable manner. In general, the manner in which the amplification target circle is associated with, or linked or coupled to, the reporter binding molecule determines the form of decoupling. For example, where the amplification target circle is base paired to a circle capture probe in the reporter binding molecule, the amplification target circle can be decoupled from the reporter binding molecule by disrupting the base pairing. Where the amplification target circle is covalently coupled to the reporter binding molecule via circle linker having a cleavable bond, the amplification target circle can be decoupled from the reporter binding molecule by cleaving the cleavable bond. To identify analytes using the amplification target circles, reporter binding molecules that are not associated with analytes should be removed prior to decoupling.

Following decoupling, the amplification target circle can be replicated by rolling circle amplification. Exponential rolling circle amplification (ERCA) is the preferred form of RCA for this purpose. If multiple different analytes are to be detected, the amplification products of amplification target circles associated with different analytes should be distinguishable. This can be accomplished in any suitable manner. For example, the amplification target circles can be in separate locations prior to decoupling and remain separated following decoupling. The separate locations could be determined, for example, by the location of the analytes with which the amplification target circles are associated. In this case, some or all of the amplification target circles can be the same (thus producing the same amplification product). The different locations of the amplification products identifies the analyte involved. As another example, some or all of the amplification target circles that are associated with different analytes can be different (thus producing different amplification products). The different amplification products identify the analytes involved. Even if the amplification target circles are mixed together and/or amplified in the same reaction, the different amplification target circles (and thus the different corresponding analytes)

can be detected and distinguished based on the differences in the amplification products.

The amplification products of RCA can be detected using any suitable technique. Real time detection, that is, detection during the RCA reaction is a preferred mode of detection with the disclosed method. Real time detection can be facilitated by use of Amplifluor™ primers. Amplifluor™ primers produce a fluorescent signal when they become incorporated into a replicated strand and are based paired with a complementary strand.

Although RCA reactions can be carried out with either linear or geometric kinetics (Lizardi et al., 1998), the disclosed method preferably uses geometric RCA. This latter form of RCA is referred to as exponential rolling circle amplification (ERCA). In exponential RCA, a secondary DNA strand displacement primer primes replication of TS-DNA to form a complementary strand referred to as secondary tandem sequence DNA or TS-DNA-2. As a secondary DNA strand displacement primer is elongated, the DNA polymerase will run into the 5' end of the next hybridized secondary DNA strand displacement molecule and will displace its 5' end. In this fashion a tandem queue of elongating DNA polymerases is formed on the TS-DNA template. As long as the rolling circle reaction continues, new secondary DNA strand displacement primers and new DNA polymerases are added to TS-DNA at the growing end of the rolling circle. A tertiary DNA strand displacement primer strand (which is complementary to the TS-DNA-2 strand and which can be the rolling circle replication primer) can then hybridize to, and prime replication of, TS-DNA-2 to form TS-DNA-3 (which is equivalent to the original TS-DNA). Strand displacement of TS-DNA-3 by the adjacent, growing TS-DNA-3 strands makes TS-DNA-3 available for hybridization with secondary DNA strand displacement primer. This results in another round of replication resulting in TS-DNA-4 (which is equivalent to TS-DNA-2). TS-DNA-4, in turn, becomes a template for DNA replication primed by tertiary DNA strand displacement primer. The cascade continues this manner until the reaction stops or reagents become limiting. The additional forms of tandem sequence DNA beyond secondary tandem sequence DNA are collectively referred to herein as higher order tandem sequence DNA. Higher order tandem sequence DNA encompasses TS-DNA-3, TS-DNA-4, and any other tandem sequence DNA produced from replication of

secondary tandem sequence DNA or the products of such replication. In a preferred mode of ERCA, the rolling circle replication primer serves as the tertiary DNA strand displacement primer, thus eliminating the need for a separate primer.

The disclosed method is preferably used to detect and analyze proteins and peptides. In preferred embodiments, multiple proteins can be analyzed using solid supports to which the various proteins are immobilized (if they are present in the sample being tested). An amplification target circle is then associated with the various proteins using a conjugate of the circle and a specific binding molecule, such as an antibody, that is specific for the protein to be detected. Rolling circle replication of the amplification target circles results in production of a large amount of DNA. The amplified DNA serves as a readily detectable signal for the proteins. Different proteins can be distinguished in several ways. For example, each different protein can be associated with a different amplification target circle that in turn is replicated to produce amplified DNA. The result is distinctive amplified DNA for each different protein. The different amplified DNAs can be distinguished using any suitable sequence-based nucleic acid detection technique. In this form of the method, many proteins can be detected in the same amplification reaction. Different amplification target circles associated with different proteins produce distinguishable amplified DNA which identifies the corresponding proteins (that is, the proteins with which the reporter binding molecules had been associated). Alternatively, the location of the amplified DNA can indicate the protein involved if different proteins are immobilized at pre-determined locations on a solid support.

Another embodiment of the disclosed method involves comparison of the proteins expressed in two or more different samples. The information generated is analogous to the type of information gathered in nucleic acid expression profiles. For example, the same analyte(s) from different samples can be associated with different amplification target circles which are replicated to produce different amplified DNAs. In this way, an analyte from one sample will produce a different amplified DNA from the same analyte in a different sample.

This sample-specific detection can be achieved even when the samples are mixed together following association of the amplification target circles with the analytes (a preferred mode of the method). For example, different analyte capture

agents can be mixed with first and second samples, respectively. This associates a different hapten with the same type of analyte in the different samples. In preferred embodiments, the samples are mixed together. The analytes can be captured on substrate, reporter binding molecules can be associated with the analyte capture agents, and DNA from the amplification target circles. Even if analytes from different samples are captured at the same location on the substrate (a preferred mode of the method), the source and amount of each analyte present at that location can be determined by virtue of the different amplified DNAs that will be produced.

The source of an analyte (that is, the sample from which the analyte came) can be determined, for example, by using different labels for different amplified DNAs (which resulted from amplification target circles keyed to the different samples). By using labels that can be distinguished when detected simultaneously with other labels (such as fluorescent labels with distinct emission spectra), all of the samples can be mixed together and analyzed together. The detected label identifies the source of the analyte indirectly through the chain of components: label to amplified DNA to circular DNA to analyte.

In another form of the disclosed method, referred to as ImmunoRCA, the amplification target circle is attached to an antibody. In one preferred form of the disclosed method, the antibody is directed against a hapten. In another preferred form of the disclosed method, the antibody is directed against the analyte itself. In the presence of a primer (referred to as a rolling circle replication primer), DNA polymerase, and nucleotides, the rolling circle reaction results in a DNA molecule consisting of multiple copies of the circle DNA sequence (referred to as tandem sequence DNA). A secondary DNA strand displacement primer is also used if exponential RCA is performed. The amplified DNA can be detected in a variety of ways, including direct incorporation of hapten- or fluorescently-labeled nucleotides, or by hybridization of fluor or enzymatically labeled complementary oligonucleotide probes.

In another aspect, the disclosed method involves immobilization of analytes present in complex biological samples and determining and quantitating their presence in the samples. For example, antigens present in biological extracts and fluids can be

identified by first selectively immobilizing them on solid supports. An immunoRCA assay can then be employed for detection and quantitation.

In another aspect, the disclosed method involves multiplexed detection and quantitation of more than one analyte in a sample. For example, a solid support can be incubated with sample containing a mixture of protein analytes to be detected, where the solid support contains immobilized capture antibodies (analyte capture agents). The solid support next can be incubated with a mixture containing at least one biotinylated antibody for each analyte. An immunoRCA microarray assay then can be employed for detection and quantitation.

In another aspect, an immunoRCA assay can be performed in microwell-glass slides, where each well is separated by a Teflon mask, or microtiter dishes. Each of the wells can be used to assay different analytes and/or different samples, and controls. Multiwell slides also can be printed with arrays of anti-IgE capture antibodies in the wells. Semi-automation of immunoRCA assays in such multiwell formats can be implemented, for example, on inexpensive liquid handling robots.

ImmunoRCA assay can be applied to other multiplexed antibody assays. For example, certain immunological reactions are caused by specific IgG_4 rather than IgE (AAAI Board of Directors, *J Allergy Clin Immunol.* 95:652-654 (1995)). The use of an anti-human IgG_4 conjugated to a DNA circle that is different in sequence from the DNA circle conjugated to an anti-IgE would allow the simultaneous measurement of allergen-specific IgG_4 and IgE. Such an assay can be used during allergen desensitisation therapy or for monitoring response to anti-IgE therapy (Chang *Nature Biotech.* 18:157-162 (2000)).

The enormous multiplexing capabilities of immunoRCA, such as the ability to detect and differentiate multiple analytes based on the sequence o f amplified DNA, can be used for clinical diagnostic tests involving detection of multiple specific antibodies, such as autoantibodies in suspected systemic autoimmune disorders, inflammatory arthritis, organ-specific autoimmune disorders or, indeed, in histocompatibility testing. Additional applications include infectious disease diagnostics with measurement of strain- and species-specific IgM and IgG, as well as *in vitro* testing of functional antibody responses in patients with suspected primary and secondary immunodeficiency diseases. Finally, the multiplexing, automation and ultrasensitivity

of this format can be applied to other immunoassays besides those involving antibody detection. RCA-powered sandwich immunoassays can provide a 8- to 9-log gain in sensitivity (signal) over conventional assays for analytes such as prostate serum antigen. Thus, the disclosed method produces a huge gain in diagnostic and prognostic power made possible by the simultaneous testing of multiple analytes for the molecular staging of disease.

Nucleic acids are ideal molecular labels for multiple analyte detection because different specific sequences can be arbitrarily associated with each individual analyte. Direct covalent coupling of nucleic acid (as a circle capture probe) to antibody permits an unlimited number of antibody-nucleic acid adducts to be prepared and used in any combination, provided that each nucleic acid is unique (Hendrickson et al., *Nucleic Acids Res.* 23: 522-529 (1995)).

Materials

A. Analytes

The disclosed method involves the detection of analytes. In general, any compound, moiety, or component of a compound or complex can be an analyte. Preferred analytes are peptides, proteins, and other macromolecules such as lipids, complex carbohydrates, proteolipids, membrane fragments, and nucleic acids. Analytes can also be smaller molecules such as cofactors, metabolites, enzyme substrates, metal ions, and metal chelates. Analytes preferably range in size from 100 daltons to 1,000,000 daltons.

Analytes may contain modifications, both naturally occurring or induced *in vitro* or *in vivo*. Induced modifications include adduct formation such as hapten attachment, multimerization, complex formation by interaction with other chemical moieties, digestion or cleavage (by, for example, protease), and metal ion attachment or removal. The disclosed method can be used to detect differences in the modification state of an analyte, such as the phosphorylation or glycosylation state of proteins.

Analytes can be associated directly or indirectly with substrates (solid supports), preferably solid supports with multiple reaction chamers. Most preferred are microtiter dishes. Analytes can be captured and/or immobilized using analyte capture agents. Immobilized analytes can be used to capture other components used in the disclosed method such as analyte capture agents and reporter binding molecules.

B. Reporter Binding Molecules

A reporter binding molecule comprises a specific binding molecule coupled or tethered to, or associated with, an amplification target circle. A reporter binding molecule can also comprise a circle capture probe, a circle linker, or both. The specific binding molecule is referred to as the affinity portion of the reporter binding molecule and the amplification target circle is referred to as the nucleic acid portion of the reporter binding molecule. The sequence of the amplification target circle sequence can be arbitrarily chosen. In a multiplex assay using multiple reporter binding molecules, it is preferred that the amplification target circle sequence for each reporter binding molecule be substantially different to limit the possibility of non-specific target detection. Alternatively, it may be desirable in some multiplex assays, to use amplification target circle sequences with related sequences. Such assays can use one or a few ATCs to detect a larger number of analytes.

Amplification target circles can be coupled or tethered to, or associated with, a specific binding molecules in any manner that allows release (decoupling) of the amplification target circles from the reporter binding molecules. For example, the amplification target circle can be base paired to a circle capture probe in the reporter binding molecule or covalently coupled to the reporter binding molecule via circle linker having a cleavable bond. As used herein, decoupling refers to physical disunion of one molecule or component from another (as for example, decoupling of an amplification target circle from a reporter binding molecule). It is specifically contemplated that decoupling refers to the physical disunion both of molecules or components that are covalent couple to each other and molecules or components that are non-covalently associated with each other. In the former case, decoupling will generally involve cleavage of one of more covalent bonds. In the latter case, decoupling will generally involve dissociation. In the case of an amplification target molecule that is tethered to a specific binding molecule, decoupling can involve dissociation, cleavage of one or more covalent bonds, or both.

A circle capture probe is an oligomer, such as an oligonucleotide, that can base pair with an amplification target circle. The region of the circle capture probe that base pairs with the amplification target circle can be any length that supports specific and stable hybridization between the circle capture probe and the amplification target circle.

Generally this is 12 to 100 nucleotides long, but is preferably 20 to 45 nucleotides long. The amplification target circle can be decoupled from the reporter binding molecule by disrupting the base pairing. In general, the circle capture probe should be incapable of priming nucleic acid synthesis. This can be accomplished in any suitable manner. For example, the circle capture probe can be coupled to the specific binding molecule via the 3' end of the circle capture probe, thus making it unavailable for extension. The 3' end of the circle capture probe can also be blocked to prevent extension. This can be accomplished by, for example, modification of the 3' end nucleotide. For example, a chemical group or molecule can be added to the 3' end. The circle capture probe can also be composed of subunits that do not support priming.

A circle linker is a component of a reporter molecule that links the amplification target circle to the specific binding molecule in a reporter binding molecule. Circle linkers preferably have a cleavable bond. As used herein, a cleavable bond is a covalent bond that can be easily and/or specifically cleaved. A cleavable bond in a circle linker is used to decouple the amplification target molecule from the reporter binding molecule. The amplification target circle can be decoupled from the reporter binding molecule by cleaving the cleavable bond.

Examples of useful circle linkers include linkers comprising a disulfide bond or a dihydroxy bond. Useful examples of linkers comprising disulfide bonds include dithiobis succinimidyl propionate, dimethyl 3,3'-dithiobispropionimidate, dithio-bis-maleimidoethane, 3,3'-dithiobis sulfosuccinimidyl propionate, succinimidyl 6-[3-(2-pyridyldithio)-propionamido]hexonate, or N-succinimidyl 3-[2-pyridyldithio]propionate. Useful examples of linkers comprising dihydroxy bonds include 1,4 bis-maleimidyl-2,3-dihydroxybutane, disuccinimidyl tartrate, or disulfosuccinimidyl tartrate. Disulfide bonds can be cleaved by, for example, treatment with a reducing agent such as β-mercaptoethanol or dithiothreitol. Dihydroxy bonds can be cleaved by, for example, treatment with periodate. Circle linkers can be attached to amplification target circles via a reactive group on the amplification target circle. Numerous reactive groups are known and can be used for this purpose. For example, the reactive group can be an allyl amino group.

Amplification target circles can be associated with or linked to specific binding molecules to form reporter binding agents before, during, or after association of the

specific binding molecule with an analyte. For example, where a specific binding molecule is coupled to a circle capture probe, the amplification target circle can be base paired with the circle capture probe after the specific binding molecule is associated with the analyte. This is illustrated in Figure 5 and Example 3. Alternatively, the amplification target circle is base paired with the circle capture probe before the specific binding molecule is associated with the analyte. This is illustrated in Figure 3 and Example 2.

Generally, an amplification target circle will be linked to a specific binding molecule through covalent coupling. That is, the specific binding molecule is covalently coupled to the circle linker, and the circle linker is covalently coupled to the amplification target circle. However, amplification target circles can also be linked to a specific binding molecule by tethering. In such a case the circle linker is the tether and is referred to as a tether circle linker. An amplification target circle is tethered to a specific binding molecule when circle linker is looped through the amplification target circle and where both sides of the circle linker (preferably both ends) are covalently coupled to the specific binding molecule. Topologically, the amplification target circle can rotate through the looped circle linker. The tether circle linker can be any material that can form a loop and be coupled to a specific binding molecule. Linear polymers are a preferred material for tether circle linkers. When the cleavable bond in the circle linker is cleaved, the tether is broken and the amplification target circle is decoupled from the reporter binding molecule.

As used herein, a specific binding molecule is a molecule that interacts specifically with a particular molecule or moiety. The molecule or moiety that interacts specifically with a specific binding molecule can be an analyte or another molecule that serves as an intermediate in the interaction between the specific binding molecule and the analyte. A preferred example of such an intermediate is an analyte capture agent. It is to be understood that the term analyte refers to both separate molecules and to portions of molecules, such as an epitope of a protein, that interacts specifically with a specific binding molecule. Antibodies, either member of a receptor/ligand pair, and other molecules with specific binding affinities are examples of specific binding molecules, useful as the affinity portion of a reporter binding molecule. A reporter binding molecule with an affinity portion that is an antibody is also referred to herein as

a reporter antibody. By coupling an amplification target circle to such specific binding molecules, binding of a specific binding molecule to its specific target can be detected by amplifying an ATC with rolling circle amplification. This amplification allows sensitive detection of a very small number of bound analytes.

A reporter binding molecule that interacts specifically with a particular analyte is said to be specific for that analyte. For example, a reporter binding molecule with an affinity portion that is an antibody that binds to a particular antigen is said to be specific for that antigen. The antigen is the analyte.

Antibodies useful as the affinity portion of reporter binding molecules, can be obtained commercially or produced using well established methods. For example, Johnstone and Thorpe, on pages 30-85, describe general methods useful for producing both polyclonal and monoclonal antibodies. The entire book describes many general techniques and principles for the use of antibodies in assay systems.

In use, the reporter binding molecules need not be absolutely pure. The reporter binding molecules preferably are at least 20% pure, more preferably at least 50% pure, more preferably at least 80% pure, and more preferably at least 90% pure.

C. Amplification Target Circles

An amplification target circle (ATC) is a circular single-stranded DNA molecule, generally containing between 40 to 1000 nucleotides, preferably between about 50 to 150 nucleotides, and most preferably between about 50 to 100 nucleotides. Portions of ATCs have specific functions making the ATC useful for rolling circle amplification (RCA). These portions are referred to as the primer complement portion, the detection tag portions, the secondary target sequence portions, the address tag portions, and the promoter portion. The primer complement portion is a required element of an amplification target circle. Detection tag portions, secondary target sequence portions, address tag portions, and promoter portions are optional. Generally, an amplification target circle is a single-stranded, circular DNA molecule comprising a primer complement portion. Those segments of the ATC that do not correspond to a specific portion of the ATC can be arbitrarily chosen sequences. It is preferred that ATCs do not have any sequences that are self-complementary. It is considered that this condition is met if there are no complementary regions greater than six nucleotides long without a mismatch or gap. It is also preferred that ATCs containing a promoter

portion do not have any sequences that resemble a transcription terminator, such as a run of eight or more thymidine nucleotides.

An amplification target circle, when replicated, gives rise to a long DNA molecule containing multiple repeats of sequences complementary to the amplification target circle. This long DNA molecule is referred to herein as tandem sequences DNA (TS-DNA). TS-DNA contains sequences complementary to the primer complement portion and, if present on the amplification target circle, the detection tag portions, the secondary target sequence portions, the address tag portions, and the promoter portion. These sequences in the TS-DNA are referred to as primer sequences (which match the sequence of the rolling circle replication primer), spacer sequences (complementary to the spacer region), detection tags, secondary target sequences, address tags, and promoter sequences. Amplification target circles are useful as components of reporter binding molecules.

D. Rolling Circle Replication Primer

A rolling circle replication primer (RCRP) is an oligonucleotide having sequence complementary to the primer complement portion of an ATC. This sequence is referred to as the complementary portion of the RCRP. The complementary portion of a RCRP and the cognate primer complement portion can have any desired sequence so long as they are complementary to each other. In general, the sequence of the RCRP can be chosen such that it is not significantly complementary to any other portion of the ATC. The complementary portion of a rolling circle replication primer can be any length that supports specific and stable hybridization between the primer and the primer complement portion. Generally this is 12 to 100 nucleotides long, but is preferably 20 to 45 nucleotides long.

It is preferred that rolling circle replication primers also contain additional sequence at the 5' end of the RCRP that is not complementary to any part of the ATC. This sequence is referred to as the non-complementary portion of the RCRP. The non-complementary portion of the RCRP, if present, serves to facilitate strand displacement during DNA replication. The non-complementary portion of a RCRP may be any length, but is generally 1 to 100 nucleotides long, and preferably 4 to 8 nucleotides long. A rolling circle replication primer can be used as the tertiary DNA strand displacement primer in exponential rolling circle amplification. For exponential rolling

circle amplification, the sequence of the rolling circle replication primer can be chosen such that it is not significantly complementary to the sequence of the secondary DNA strand displacement primer.

In preferred embodiments, rolling circle replication primers (and other primers used in the method) can contain a spacer. The spacer can help to overcome steric factors from the surface when immobilized, aid in anchoring polymerase on primers, or provide other advantages, such as control or alteration of the hydrophobicity of elements attached to a solid support. Spacers useful for the disclosed method include nucleotide spacers such as poly dT or poly dA; aliphatic linkers such as C18, C12, or multimers thereof; aromatic spacers, or RNA, DNA, PNA or combinations thereof.

Rolling circle replication primers are preferably Amplifluor™ primers. Amplifluors™ are fluorescent moieties and quenchers incorporated into primers containing stem structures (usually in hairpin or stem and loop structures) such that the quencher moiety is in proximity with the fluorescent moiety. That is, the quencher and fluorescent are incorporated into opposite strands of the stem structure. In the structured state, the quencher prevents or limits fluorescence of the fluorescent moiety. When the stem of the primer is disrupted, the quencher and fluorescent moiety are no longer in proximity and the fluorescent moiety produces a fluorescent signal. In the disclosed method, use of Amplifluor™ primers in ERCA produces double stranded tandem sequence DNA where the primer stem is disrupted in favor of a complementary, replicated strand. From a reaction initially containing structured (that is, non-fluorescent) Amplifluor™ primers, fluorescence signal increases as amplification takes place, as more and more of the Amplifluor™ primers are incorporated into double stranded TS-DNA, as the Amplifluor™ stems are disrupted, and as the fluorescent moieties as consequently unquenched. Thus, use of Amplifluor™ primers is particularly suited for real-time detection of amplification in ERCA. Amplifluor™ primers are also referred to herein as fluorescent quenched primers. Thus, an Amplifluor™ rolling circle replication primer is also referred to as a fluorescent quenched rolling circle replication primer.

E. Analyte Capture Agents

An analyte capture agent is any compound that can interact with an analyte and allow the analyte to be immobilized or separated from other compounds and analytes.

An analyte capture agent includes an analyte interaction portion. Analyte capture agents can also include a capture portion. Analyte capture agents without a capture portion preferably are immobilized on a solid support. The analyte interaction portion of an analyte capture agent is a molecule that interacts specifically with a particular molecule or moiety. The molecule or moiety that interacts specifically with an analyte interaction portion can be an analyte or another molecule that serves as an intermediate in the interaction between the analyte interaction portion and the analyte. It is to be understood that the term analyte refers to both separate molecules and to portions of molecules, such as an epitope of a protein, that interacts specifically with an analyte interaction portion. Antibodies, either member of a receptor/ligand pair, and other molecules with specific binding affinities are examples of molecules that can be used as an analyte interaction portion of an analyte capture agent. The analyte interaction portion of an analyte capture agent can also be any compound or composition with which an analyte can interact, such as peptides. An analyte capture agent that interacts specifically with a particular analyte is said to be specific for that analyte. For example, an analyte capture agent with an analyte interaction portion that is an antibody that binds to a particular antigen is said to be specific for that antigen. The antigen is the analyte.

Examples of molecules useful as the analyte interaction portion of analyte capture agents are antibodies, such as crude (serum) antibodies, purified antibodies, monoclonal antibodies, polyclonal antibodies, synthetic antibodies, antibody fragments (for example, Fab fragments); antibody interacting agents, such as protein A, carbohydrate binding proteins, and other interactants; protein interactants (for example avidin and its derivatives); peptides; and small chemical entities, such as enzyme substrates, cofactors, metal ions/chelates, and haptens. Antibodies may be modified or chemically treated to optimize binding to surfaces and/or targets.

Antibodies useful as the analyte interaction portion of analyte capture agents, can be obtained commercially or produced using well-established methods. For example, Johnstone and Thorpe, on pages 30-85, describe general methods useful for producing both polyclonal and monoclonal antibodies. The entire book describes many general techniques and principles for the use of antibodies in assay systems.

The capture portion of an analyte capture agent is any compound that can be associated with another compound. Preferably, a capture portion is a compound, such as a ligand or hapten, that binds to or interacts with another compound, such as ligand-binding molecule or an antibody. It is also preferred that such interaction between the capture portion and the capturing component be a specific interaction, such as between a hapten and an antibody or a ligand and a ligand-binding molecule. Examples of haptens include biotin, FITC, digoxigenin, and dinitrophenol. The capture portion can be used to separate compounds or complexes associated with the analyte capture agent from those that do not.

Capturing analytes or analyte capture agents on a substrate may be accomplished in several ways. In one embodiment, capture docks are adhered or coupled to the substrate. Capture docks are compounds or moieties that mediate adherence of an analyte by binding to, or interacting with, the capture portion on an analyte capture agent (with which the analyte is, or will be, associated). Capture docks immobilized on a substrate allow capture of the analyte on the substrate. Such capture provides a convenient means of washing away reaction components that might interfere with subsequent steps. Alternatively, analyte capture agents can be directly immobilized on a substrate. In this case, the analyte capture agent need not have a capture portion.

In one embodiment, the analyte capture agent or capture dock to be immobilized is an anti-hybrid antibody. Methods for immobilizing antibodies and other proteins to substrates are well established. Immobilization can be accomplished by attachment, for example, to aminated surfaces, carboxylated surfaces or hydroxylated surfaces using standard immobilization chemistries. Examples of attachment agents are cyanogen bromide, succinimide, aldehydes, tosyl chloride, avidin-biotin, photocrosslinkable agents, epoxides and maleimides. A preferred attachment agent is a heterobifunctional cross-linking agent such as N-[γ-maleimidobutyryloxy]succinimide ester (GMBS). These and other attachment agents, as well as methods for their use in attachment, are described in *Protein immobilization: fundamentals and applications*, Richard F. Taylor, ed. (M. Dekker, New York, 1991), Johnstone and Thorpe, *Immunochemistry In Practice* (Blackwell Scientific Publications, Oxford, England, 1987) pages 209-216 and 241-242, and *Immobilized*

Affinity Ligands, Craig T. Hermanson *et al.*, eds. (Academic Press, New York, 1992). Antibodies can be attached to a substrate by chemically cross-linking a free amino group on the antibody to reactive side groups present within the substrate. For example, antibodies may be chemically cross-linked to a substrate that contains free amino, carboxyl, or sulfur groups using glutaraldehyde, carbodiimides, or heterobifunctional agents such as GMBS as cross-linkers. In this method, aqueous solutions containing free antibodies are incubated with the solid support in the presence of glutaraldehyde or carbodiimide. For crosslinking with glutaraldehyde the reactants can be incubated with 2% glutaraldehyde by volume in a buffered solution such as 0.1 M sodium cacodylate at pH 7.4. Other standard immobilization chemistries are known by those of skill in the art.

One useful form of analyte capture agents are peptides. When various peptides are immobilized on a solid support, they can be used as "bait" for analytes. For example, a set of different peptides on a solid support can be used to access whether a sample has analytes that interact with any of the peptides. Comparisons of different samples can be made by, for example, noting differences in the peptides to which analytes in the different samples become associated. In another form of the disclosed method, a set of analyte capture agents specific for analytes of interest can be used to access the presence of a whole suite of analytes in a sample.

In use, the analyte capture agents need not be absolutely pure. The analyte capture agents preferably are at least 20% pure, more preferably at least 50% pure, more preferably at least 80% pure, and more preferably at least 90% pure.

F. Accessory Molecules

Accessory molecules are molecules that affect the interaction of analytes and specific binding molecules or analyte capture agents. For example, accessory molecules can be molecules that compete with the binding of an analyte with an analyte capture agent or specific binding molecule. One form of competitive accessory molecules are analogs of analytes. An analog is a molecule that is similar in structure but different in competition. In this context, the analyte analog should be sufficiently similar to interact with an analyte capture agent or specific binding molecule specific for that analyte. Accessory molecules can also be molecules that aid or are necessary

for interaction of an analyte and a specific binding molecule or analyte capture agent. Such accessory molecules are referred to herein as analyte binding co-factors.

In one form of the disclosed method, accessory molecules can be compounds that are to be tested for their effect on analyte binding. For example, the disclosed method can be used to screen for competitors (or binding co-factors) of an analyte interaction with a specific binding molecule or analyte capture agent. If an accessory molecule affects interaction of the analyte, the results of RCA will change since the association of the reporter binding molecule to the analyte (or of the analyte capture agent to the analyte) will be lost or gained.

In use, the accessory molecules need not be absolutely pure. The accessory molecules preferably are at least 20% pure, more preferably at least 50% pure, more preferably at least 80% pure, and more preferably at least 90% pure.

G. Detection Labels

To aid in detection and quantitation of nucleic acids amplified using the disclosed method, detection labels can be directly incorporated into amplified nucleic acids or can be coupled to detection molecules. As used herein, a detection label is any molecule that can be associated with amplified nucleic acid, directly or indirectly, and which results in a measurable, detectable signal, either directly or indirectly. Many such labels for incorporation into nucleic acids or coupling to nucleic acid probes are known to those of skill in the art. Examples of detection labels suitable for use in the disclosed method are radioactive isotopes, fluorescent molecules, phosphorescent molecules, enzymes, antibodies, and ligands.

Examples of suitable fluorescent labels include fluorescein isothiocyanate (FITC), 5,6-carboxymethyl fluorescein, Texas red, nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), coumarin, dansyl chloride, rhodamine, amino-methyl coumarin (AMCA), Eosin, Erythrosin, BODIPY®, Cascade Blue®, Oregon Green®, pyrene, lissamine, xanthenes, acridines, oxazines, phycoerythrin, macrocyclic chelates of lanthanide ions such as quantum dye™, fluorescent energy transfer dyes, such as thiazole orange-ethidium heterodimer, and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. Examples of other specific fluorescent labels include 3-Hydroxypyrene 5,8,10-Tri Sulfonic acid, 5-Hydroxy Tryptamine (5-HT), Acid Fuchsin, Alizarin Complexon, Alizarin Red, Allophycocyanin, Aminocoumarin, Anthroyl Stearate, Astrazon Brilliant

Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, Auramine, Aurophosphine, Aurophosphine G, BAO 9 (Bisaminophenyloxadiazole), BCECF, Berberine Sulphate, Bisbenzamide, Blancophor FFG Solution, Blancophor SV, Bodipy F1, Brilliant Sulphoflavin FF, Calcien Blue, Calcium Green, Calcofluor RW Solution, Calcofluor White, Calcophor White ABT Solution, Calcophor White Standard Solution, Carbostyryl, Cascade Yellow, Catecholamine, Chinacrine, Coriphosphine O, Coumarin-Phalloidin, CY3.1 8, CY5.1 8, CY7, Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid), Dansa (Diamino Naphtyl Sulphonic Acid), Dansyl NH-CH_3, Diamino Phenyl Oxydiazole (DAO), Dimethylamino-5-Sulphonic acid, Dipyrrometheneboron Difluoride, Diphenyl Brilliant Flavine 7GFF, Dopamine, Erythrosin ITC, Euchrysin, FIF (Formaldehyde Induced Fluorescence), Flazo Orange, Fluo 3, Fluorescamine, Fura-2, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow 5GF, Gloxalic Acid, Granular Blue, Haematoporphyrin, Indo-1, Intrawhite Cf Liquid, Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine Rhodamine B200 (RD200), Lucifer Yellow CH, Lucifer Yellow VS, Magdala Red, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, MPS (Methyl Green Pyronine Stilbene), Mithramycin, NBD Amine, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant Flavin E8G, Oxadiazole, Pacific Blue, Pararosaniline (Feulgen), Phorwite AR Solution, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, Phthalocyanine, Phycoerythrin R, Polyazaindacene Pontochrome Blue Black, Porphyrin, Primuline, Procion Yellow, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, Quinacrine Mustard, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B Extra, Rhodamine BB, Rhodamine BG, Rhodamine WT, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS (Primuline), SITS (Stilbene Isothiosulphonic acid), Stilbene, Snarf 1, sulpho Rhodamine B Can C, Sulpho Rhodamine G Extra, Tetracycline, Thiazine Red R, Thioflavin S, Thioflavin TCN, Thioflavin 5, Thiolyte, Thiozol Orange, Tinopol CBS, True Blue, Ultralite, Uranine B, Uvitex SFC, Xylene Orange, and XRITC.

Preferred fluorescent labels are fluorescein (5-carboxyfluorescein-N-hydroxysuccinimide ester), rhodamine (5,6-tetramethyl rhodamine), and the cyanine

dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. The absorption and emission maxima, respectively, for these fluors are: FITC (490 nm; 520 nm), Cy3 (554 nm; 568 nm), Cy3.5 (581 nm; 588 nm), Cy5 (652 nm: 672 nm), Cy5.5 (682 nm; 703 nm) and Cy7 (755 nm; 778 nm), thus allowing their simultaneous detection. Other examples of fluorescein dyes include 6-carboxyfluorescein (6-FAM), 2',4',1,4,-tetrachlorofluorescein (TET), 2',4',5',7',1,4-hexachlorofluorescein (HEX), 2',7'-dimethoxy-4', 5'-dichloro-6-carboxyrhodamine (JOE), 2'-chloro-5'-fluoro-7',8'-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED), and 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC). Fluorescent labels can be obtained from a variety of commercial sources, including Amersham Pharmacia Biotech, Piscataway, NJ; Molecular Probes, Eugene, OR; and Research Organics, Cleveland, Ohio.

Additional labels of interest include those that provide for signal only when the probe with which they are associated is specifically bound to a target molecule. Such labels include "molecular beacons" as described in Tyagi & Kramer, Nature Biotechnology (1996) 14:303 and EP 0 070 685 B1. Other labels of interest include those described in U.S. Pat. No. 5,563,037; WO 97/17471 and WO 97/17076.

Another useful label, related to molecular beacon technology, are Amplifluors™. Amplifluors™ are fluorescent moieties and quenchers incorporated into primers containing stem structures (usually in hairpin or stem and loop structures) such that the quencher moiety is in proximity with the fluorescent moiety. That is, the quencher and fluorescent are incorporated into opposite strands of the stem structure. In the structured state, the quencher prevents or limits fluorescence of the fluorescent moiety. When the stem of the primer is disrupted, the quencher and fluorescent moiety are no longer in proximity and the fluorescent moiety produces a fluorescent signal. In the disclosed method, use of Amplifluor™ primers in ERCA produces double stranded tandem sequence DNA where the primer stem is disrupted in favor of a complementary, replicated strand. From a reaction initially containing structured (that is, non-fluorescent) Amplifluor™ primers, fluorescence signal increases as amplification takes place, as more and more of the Amplifluor™ primers are incorporated into double stranded TS-DNA, as the Amplifluor™ stems are disrupted, and as the fluorescent moieties as consequently unquenched. Thus, use of Amplifluors™ is particularly suited for real-time detection of amplification in ERCA.

Labeled nucleotides are a preferred form of detection label since they can be directly incorporated into the amplification products during synthesis. Examples of detection labels that can be incorporated into amplified nucleic acids include nucleotide analogs such as BrdUrd (5-bromodeoxyuridine, Hoy and Schimke, *Mutation Research* **290**:217-230 (1993)), aminoallyldeoxyuridine (Henegariu *et al., Nature Biotechnology* **18**:345-348 (2000)), 5-methylcytosine (Sano *et al., Biochim. Biophys. Acta* **951**:157-165 (1988)), bromouridine (Wansick *et al., J. Cell Biology* **122**:283-293 (1993)) and nucleotides modified with biotin (Langer *et al., Proc. Natl. Acad. Sci. USA* **78**:6633 (1981)) or with suitable haptens such as digoxygenin (Kerkhof, *Anal. Biochem.* **205**:359-364 (1992)). Suitable fluorescence-labeled nucleotides are Fluorescein-isothiocyanate-dUTP, Cyanine-3-dUTP and Cyanine-5-dUTP (Yu *et al., Nucleic Acids Res.*, **22**:3226-3232 (1994)). A preferred nucleotide analog detection label for DNA is BrdUrd (bromodeoxyuridine, BrdUrd, BrdU, BUdR, Sigma-Aldrich Co). Other useful nucleotide analogs for incorporation of detection label into DNA are AA-dUTP (aminoallyl-deoxyuridine triphosphate, Sigma-Aldrich Co.), and 5-methyl-dCTP (Roche Molecular Biochemicals). A preferred nucleotide analog for incorporation of detection label into RNA is biotin-16-UTP (biotin-16-uridine-5'-triphosphate, Roche Molecular Biochemicals). Fluorescein, Cy3, and Cy5 can be linked to dUTP for direct labeling. Cy3.5 and Cy7 are available as avidin or anti-digoxygenin conjugates for secondary detection of biotin- or digoxygenin-labeled probes.

Detection labels that are incorporated into amplified nucleic acid, such as biotin, can be subsequently detected using sensitive methods known in the art. For example, biotin can be detected using streptavidin-alkaline phosphatase conjugate (Tropix, Inc.), which is bound to the biotin and subsequently detected by chemiluminescence of suitable substrates (for example, chemiluminescent substrate CSPD: disodium, 3-(4-methoxyspiro-[1,2,-dioxetane-3-2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decane]-4-yl) phenyl phosphate; Tropix, Inc.). Labels can also be enzymes, such as alkaline phosphatase, soybean peroxidase, horseradish peroxidase and polymerases, that can be detected, for example, with chemical signal amplification or by using a substrate to the enzyme which produces light (for example, a chemiluminescent 1,2-dioxetane substrate) or fluorescent signal.

Molecules that combine two or more of these detection labels are also considered detection labels. Any of the known detection labels can be used with the disclosed probes, tags, and method to label and detect nucleic acid amplified using the disclosed method. Methods for detecting and measuring signals generated by detection labels are also known to those of skill in the art. For example, radioactive isotopes can be detected by scintillation counting or direct visualization; fluorescent molecules can be detected with fluorescent spectrophotometers; phosphorescent molecules can be detected with a spectrophotometer or directly visualized with a camera; enzymes can be detected by detection or visualization of the product of a reaction catalyzed by the enzyme; antibodies can be detected by detecting a secondary detection label coupled to the antibody. As used herein, detection molecules are molecules which interact with amplified nucleic acid and to which one or more detection labels are coupled.

H. Detection Probes

Detection probes are labeled oligonucleotides having sequence complementary to detection tags on TS-DNA. The complementary portion of a detection probe can be any length that supports specific and stable hybridization between the detection probe and the detection tag. For this purpose, a length of 10 to 35 nucleotides is preferred, with a complementary portion of a detection probe 16 to 20 nucleotides long being most preferred. Detection probes can contain any of the detection labels described above. Preferred labels are biotin and fluorescent molecules. A particularly preferred detection probe is a molecular beacon. Molecular beacons are detection probes labeled with fluorescent moieties where the fluorescent moieties fluoresce only when the detection probe is hybridized (Tyagi and Kramer, *Nature Biotechnology* **14**:303-308 (1996)). The use of such probes eliminates the need for removal of unhybridized probes prior to label detection because the unhybridized detection probes will not produce a signal. This is especially useful in multiplex assays. The TS-DNA can be collapsed as described in WO 97/19193 using collapsing detection probes. Collapsing TS-DNA is especially useful with combinatorial multicolor coding, which is described below.

I. DNA Strand Displacement Primers

Primers used for secondary DNA strand displacement (an example of which is exponential rolling circle amplification) are referred to herein as DNA strand

displacement primers. One form of DNA strand displacement primer, referred to herein as a secondary DNA strand displacement primer, is an oligonucleotide having sequence matching part of the sequence of an ATC. This sequence is referred to as the matching portion of the secondary DNA strand displacement primer. This matching portion of a secondary DNA strand displacement primer is complementary to sequences in TS-DNA. The matching portion of a secondary DNA strand displacement primer may be complementary to any sequence in TS-DNA. The matching portion of a secondary DNA strand displacement primer can be any length that supports specific and stable hybridization between the primer and its complement. Generally this is 12 to 35 nucleotides long, but is preferably 18 to 25 nucleotides long. In general, the sequence of a secondary DNA strand displacement primer should be chosen such that it is not significantly complementary to the sequence of the rolling circle replication primer with which it is used. Secondary DNA strand displacement primers are used with tertiary strand displacement primers in exponential rolling circle amplification. In general, the sequence of a secondary DNA strand displacement primer should be chosen such that it is not significantly complementary to the sequence of the tertiary DNA strand displacement primer with which it is used.

Another form of DNA strand displacement primer, referred to herein as a tertiary DNA strand displacement primer, is an oligonucleotide having sequence complementary to part of the sequence of an ATC. This sequence is referred to as the complementary portion of the tertiary DNA strand displacement primer. This complementary portion of the tertiary DNA strand displacement primer matches sequences in TS-DNA. The complementary portion of a tertiary DNA strand displacement primer may be complementary to any sequence in the ATC. The complementary portion of a tertiary DNA strand displacement primer can be any length that supports specific and stable hybridization between the primer and its complement. Generally this is 12 to 35 nucleotides long, but is preferably 18 to 25 nucleotides long. In general, the sequence of a tertiary DNA strand displacement primer should be chosen such that it is not significantly complementary to the sequence of the secondary DNA strand displacement primer with which it is used. A preferred tertiary DNA strand displacement primer is a rolling circle replication primer. In this case, the sequence of the rolling circle replication primer should be chosen such that it is not

significantly complementary to the sequence of the secondary DNA strand displacement primer with which it is used. DNA strand displacement primers and their use are described in more detail in U.S. Patent No. 5,854,033 and WO 97/19193.

DNA strand displacement primers preferably are Amplifluor™ primers. In the disclosed method, use of Amplifluor™ primers in ERCA produces double stranded tandem sequence DNA where the primer stem is disrupted in favor of a complementary, replicated strand. From a reaction initially containing structured (that is, non-fluorescent) Amplifluor™ primers, fluorescence signal increases as amplification takes place, as more and more of the Amplifluor™ primers are incorporated into double stranded TS-DNA, as the Amplifluor™ stems are disrupted, and as the fluorescent moieties as consequently unquenched. Thus, use of Amplifluors™ is particularly suited for real-time detection of amplification in ERCA. If Amplifluor™ primers are used, only one of the primers in a RCA reaction need be an Amplifluor™ primer. However, any or all of the primers used can be Amplifluor™ primers, and any combination of Amplifluor™ and non-Amplifluor™ primers can be used. For example, the rolling circle replication primer can be non-Amplifluor™ while the secondary DNA strand displacement primer can be Amplifluor™, or the rolling circle replication primers can be a mixture of Amplifluor™ and non-Amplifluor™ primers. Amplifluor™ primers are also referred to herein as fluorescent quenched primers. Thus, an Amplifluor™ DNA strand displacement primer is also referred to as a fluorescent quenched DNA strand displacement primer.

J. Oligonucleotide Synthesis

Rolling circle replication primers, circle capture probes, circle linkers, detection probes, address probes, amplification target circles, DNA strand displacement primers, and any other oligonucleotides can be synthesized using established oligonucleotide synthesis methods. Methods to produce or synthesize oligonucleotides are well known in the art. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method. Solid phase chemical synthesis of DNA fragments is routinely performed using protected nucleoside cyanoethyl

phosphoramidites (S. L. Beaucage et al. (1981) Tetrahedron Lett. 22:1859). In this approach, the 3'-hydroxyl group of an initial 5'-protected nucleoside is first covalently attached to the polymer support (R. C. Pless et al. (1975) Nucleic Acids Res. 2:773 (1975)). Synthesis of the oligonucleotide then proceeds by deprotection of the 5'-hydroxyl group of the attached nucleoside, followed by coupling of an incoming nucleoside-3'-phosphoramidite to the deprotected hydroxyl group (M. D. Matteucci et a. (1981) J. Am. Chem. Soc. 103:3185). The resulting phosphite triester is finally oxidized to a phosphorotriester to complete the internucleotide bond (R. L. Letsinger et al. (1976) J. Am. Chem. Soc. 9:3655). Alternatively, the synthesis of phosphorothioate linkages can be carried out by sulfurization of the phosphite triester. Several chemicals can be used to perform this reaction, among them 3H-1,2-benzodithiole-3-one, 1,1-dioxide (R.P. Iyer, W. Egan, J.B. Regan, and S.L. Beaucage, J. Am. Chem. Soc., 1990, 112, 1253-1254). The steps of deprotection, coupling and oxidation are repeated until an oligonucleotide of the desired length and sequence is obtained. Other methods exist to generate oligonucleotides such as the H-phosphonate method (Hall et al, (1957) J. Chem. Soc., 3291-3296) or the phosphotriester method as described by Ikuta *et al.*, *Ann. Rev. Biochem.* **53**:323-356 (1984), (phosphotriester and phosphite-triester methods), and Narang *et al.*, *Methods Enzymol.*, **65**:610-620 (1980), (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen *et al.*, *Bioconjug. Chem.* **5**:3-7 (1994). Other forms of oligonucleotide synthesis are described in U.S. Patent No. 6,294,664 and U.S. Patent No. 6,291,669.

Many of the oligonucleotides described herein are designed to be complementary to certain portions of other oligonucleotides or nucleic acids such that stable hybrids can be formed between them via base pairing. The stability of these hybrids can be calculated using known methods such as those described in Lesnick and Freier, *Biochemistry* **34**:10807-10815 (1995), McGraw *et al.*, *Biotechniques* **8**:674-678 (1990), and Rychlik *et al.*, *Nucleic Acids Res.* **18**:6409-6412 (1990).

Oligonucleotides can be synthesized, for example, on a Perseptive Biosystems 8909 Expedite Nucleic Acid Synthesis system using standard β-cyanoethyl phosphoramidite coupling chemistry on synthesis columns (Glen Research, Sterling, VA). Oxidation of the newly formed phosphites can be carried out using, for example,

the sulfurizing reagent 3H-1,2-benzothiole-3-one-1,1-idoxide (Glen Research) or the standard oxidizing reagent after the first and second phosphoramidite addition steps. The thio-phosphitylated oligonucleotides can be deprotected, for example, using 30% ammonium hydroxide (3.0 ml) in water at 55°C for 16 hours, concentrated in an OP 120 Savant Oligo Prep deprotection unit for 2 hours, and desalted with PD10 Sephadex columns using the protocol provided by the manufacturer.

So long as their relevant function is maintained, rolling circle replication primers, circle capture probes, circle linkers, detection probes, address probes, amplification target circles, DNA strand displacement primers, and any other oligonucleotides can be made up of or include modified nucleotides (nucleotide analogs). Many modified nucleotides are known and can be used in oligonucleotides. A nucleotide analog is a nucleotide which contains some type of modification to either the base, sugar, or phosphate moieties. Modifications to the base moiety would include natural and synthetic modifications of A, C, G, and T/U as well as different purine or pyrimidine bases, such as uracil-5-yl, hypoxanthin-9-yl (I), and 2-aminoadenin-9-yl. A modified base includes but is not limited to 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Additional base modifications can be found for example in U.S. Pat. No. 3,687,808, Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B. ed., CRC Press, 1993. Certain nucleotide analogs, such as 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine can increase the stability of duplex formation. Other modified bases are those that function as universal bases.

Universal bases include 3-nitropyrrole and 5-nitroindole. Universal bases substitute for the normal bases but have no bias in base pairing. That is, universal bases can base pair with any other base. Base modifications often can be combined with for example a sugar modification, such as 2'-O-methoxyethyl, to achieve unique properties such as increased duplex stability. There are numerous United States patents such as 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, which detail and describe a range of base modifications. Each of these patents is herein incorporated by reference.

Nucleotide analogs can also include modifications of the sugar moiety. Modifications to the sugar moiety would include natural modifications of the ribose and deoxyribose as well as synthetic modifications. Sugar modifications include but are not limited to the following modifications at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C1 to C10, alkyl or C2 to C10 alkenyl and alkynyl. 2' sugar modifications also include but are not limited to -O[(CH_2)n O]m CH_3, -O(CH_2)n OCH_3, -O(CH_2)n NH_2, -O(CH_2)n CH_3, -O(CH_2)n -ONH_2, and -O(CH_2)nON[(CH_2)n $CH_3)]_2$, where n and m are from 1 to about 10.

Other modifications at the 2' position include but are not limited to: C1 to C10 lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2 CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Similar modifications may also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Modified sugars would also include those that contain modifications at the bridging ring oxygen, such as CH_2 and S. Nucleotide sugar analogs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. There are numerous United States patents that teach the preparation of such modified sugar structures such as 4,981,957;

5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference in its entirety.

Nucleotide analogs can also be modified at the phosphate moiety. Modified phosphate moieties include but are not limited to those that can be modified so that the linkage between two nucleotides contains a phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, methyl and other alkyl phosphonates including 3'-alkylene phosphonate and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates. It is understood that these phosphate or modified phosphate linkages between two nucleotides can be through a 3'-5' linkage or a 2'-5' linkage, and the linkage can contain inverted polarity such as 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. Numerous United States patents teach how to make and use nucleotides containing modified phosphates and include but are not limited to, 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

It is understood that nucleotide analogs need only contain a single modification, but may also contain multiple modifications within one of the moieties or between different moieties.

Nucleotide substitutes are molecules having similar functional properties to nucleotides, but which do not contain a phosphate moiety, such as peptide nucleic acid (PNA). Nucleotide substitutes are molecules that will recognize and hybridize to (base pair to) complementary nucleic acids in a Watson-Crick or Hoogsteen manner, but which are linked together through a moiety other than a phosphate moiety. Nucleotide substitutes are able to conform to a double helix type structure when interacting with the appropriate target nucleic acid.

Nucleotide substitutes are nucleotides or nucleotide analogs that have had the phosphate moiety and/or sugar moieties replaced. Nucleotide substitutes do not contain a standard phosphorus atom. Substitutes for the phosphate can be for example, short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH2 component parts. Numerous United States patents disclose how to make and use these types of phosphate replacements and include but are not limited to 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

It is also understood in a nucleotide substitute that both the sugar and the phosphate moieties of the nucleotide can be replaced, by for example an amide type linkage (aminoethylglycine) (PNA). United States patents 5,539,082; 5,714,331; and 5,719,262 teach how to make and use PNA molecules, each of which is herein incorporated by reference. (See also Nielsen *et al.*, *Science* 254:1497-1500 (1991)).

Oligonucleotides can be comprised of nucleotides and can be made up of different types of nucleotides or the same type of nucleotides. For example, one or more of the nucleotides in an oligonucleotide can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; about 10% to about 50% of the nucleotides can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; about 50% or more of the nucleotides can be ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides; or all of the nucleotides

are ribonucleotides, 2'-O-methyl ribonucleotides, or a mixture of ribonucleotides and 2'-O-methyl ribonucleotides.

K. Solid Supports

Solid supports are solid-state substrates or supports with which analytes (or other components used in the disclosed method) can be associated. Analytes can be associated with solid supports directly or indirectly. For example, analytes can be directly immobilized on solid supports. Analyte capture agents and accessory molecules can also be immobilized on solid supports. A preferred form of solid support is a microtiter dish. Another form of solid support is an array detector. An array detector is a solid support to which multiple different address probes or detection molecules have been coupled in an array, grid, or other organized pattern.

Rolling circle amplification of decoupled amplification target circles can be performed on solid supports having reaction chambers. A reaction chamber is any structure in which a separate amplification reaction can be performed. Useful reaction chambers include wells, vessels, tubes, chambers, holes, depressions, dimples, locations, or other structures that can support separate reactions. Solid supports preferably comprise arrays of reaction chambers. In connection with reaction chambers, a separate reaction refers to a reaction where substantially no cross contamination of reactants or products will occur between different reaction chambers. Substantially no cross contamination refers to a level of contamination of reactants or products below a level that would be detected in the particular reaction or assay involved. For example, if TS-DNA contamination from another reaction chamber would not be detected in a given reaction chamber in a given assay (even though it may be present), there is no substantial cross contamination of the TS-DNA. It is understood, therefore, that reaction chambers can comprise, for example, locations on a planar surface so long as the reactions performed at the locations remain separate and are not subject to mixing.

Solid-state substrates for use in solid supports can include any solid material with which analytes can be associated, directly or indirectly. This includes materials such as acrylamide, agarose, cellulose, cellulose, nitrocellulose, glass, gold, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, glass, polysilicates, polycarbonates, teflon,

fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, functionalized silane, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Solid-state substrates can have any useful form including thin film, membrane, bottles, dishes, fibers, woven fibers, shaped polymers, particles, beads, microparticles, or a combination. Solid-state substrates and solid supports can be porous or non-porous. A preferred form for a solid-state substrate is a microtiter dish. The most preferred form of microtiter dish is the standard 96-well type. In some embodiments, a multiwell glass slide can be employed.

Different analytes, analyte capture agents, or accessory molecules can be used together as a set. The set can be used as a mixture of all or subsets of the analytes, analyte capture agents, and accessory molecules used separately in separate reactions, or immobilized on a solid support. Analytes, analyte capture agents, and accessory molecules used separately or as mixtures can be physically separable through, for example, association with or immobilization on a solid support. An array includes a plurality of analytes, analyte capture agents and/or accessory molecules immobilized at identified or predefined locations on the solid support. Each predefined location on the solid support generally has one type of component (that is, all the components at that location are the same). Alternatively, multiple types of components can be immobilized in the same predefined location on a solid support. Each location will have multiple copies of the given components. The spatial separation of different components on the solid support allows separate detection and identification of analytes.

Although preferred, it is not required that the solid support be a single unit or structure. The set of analytes, analyte capture agents, or accessory molecules may be distributed over any number of solid supports. For example, at one extreme, each probe may be immobilized in a separate reaction tube or container, or on separate beads or microparticles. Different modes of the disclosed method can be performed with different components (for example, analytes, analyte capture agents, and accessory molecules) immobilized on a solid support.

In alternative embodiments, RCA is performed in solution, and the products of the amplification are captured on a solid support. For example, the decoupled amplification target circles can be amplified together (that is, not in separate reaction

chambers) and the products captured. For example, a biotinylated capture antibody can be added to a sample containing the analyte, followed by a reporter binding molecule that binds to a different location on the analyte. These components--the capture antibody and the reporter binding molecule--can be added in any order. RCA then can be performed to produce TS-DNA, and purified on a matrix containing streptavidin (streptavidin beads (Dynal), for example). The TS-DNA then can be detected or quantitated by hybridization to a solid support containing oligonucleotide probes complementary to the TS-DNA. Such probes are referred to herein as address probes. By attaching different address probes to different regions of a solid support, different RCA products can be captured at different, and therefore diagnostic, locations on the solid support. For example, in a microtiter plate multiplex assay, address probes specific for up to 96 different TS-DNAs (each amplified via different primers and ATCs) can be immobilized on a microtiter plate, each in a different well. Capture and detection will occur only in those probe elements on the solid support corresponding to TS-DNAs for which the corresponding analytes were present in a sample.

Methods for immobilization of oligonucleotides to solid-state substrates are well established. Oligonucleotides, including address probes and detection probes, can be coupled to substrates using established coupling methods. For example, suitable attachment methods are described by Pease *et al.*, *Proc. Natl. Acad. Sci. USA* **91**(11):5022-5026 (1994), and Khrapko *et al.*, *Mol Biol (Mosk) (USSR)* 25:718-730 (1991). A method for immobilization of 3'-amine oligonucleotides on casein-coated slides is described by Stimpson *et al.*, *Proc. Natl. Acad. Sci. USA* **92**:6379-6383 (1995). A preferred method of attaching oligonucleotides to solid-state substrates is described by Guo *et al.*, *Nucleic Acids Res.* 22:5456-5465 (1994).

Some solid supports useful in RCA assays have detection antibodies attached to a solid-state substrate. Such antibodies can be specific for a molecule of interest. Captured molecules of interest can then be detected by binding of a second, reporter antibody, followed by RCA. Such a use of antibodies in a solid support allows RCA assays to be developed for the detection of any molecule for which antibodies can be generated. Methods for immobilizing antibodies to solid-state substrates are well established. Immobilization can be accomplished by attachment, for example, to aminated surfaces, carboxylated surfaces or hydroxylated surfaces using standard

immobilization chemistries. Examples of attachment agents are cyanogen bromide, succinimide, aldehydes, tosyl chloride, avidin-biotin, photocrosslinkable agents, epoxides and maleimides. A preferred attachment agent is the heterobifunctional cross-linker N-[γ-Maleimidobutyryloxy] succinimide ester (GMBS). These and other attachment agents, as well as methods for their use in attachment, are described in *Protein immobilization: fundamentals and applications*, Richard F. Taylor, ed. (M. Dekker, New York, 1991), Johnstone and Thorpe, *Immunochemistry In Practice* (Blackwell Scientific Publications, Oxford, England, 1987) pages 209-216 and 241-242, and *Immobilized Affinity Ligands*, Craig T. Hermanson *et al.*, eds. (Academic Press, New York, 1992). Antibodies can be attached to a substrate by chemically cross-linking a free amino group on the antibody to reactive side groups present within the solid-state substrate. For example, antibodies may be chemically cross-linked to a substrate that contains free amino, carboxyl, or sulfur groups using glutaraldehyde, carbodiimides, or GMBS, respectively, as cross-linker agents. In this method, aqueous solutions containing free antibodies are incubated with the solid-state substrate in the presence of glutaraldehyde or carbodiimide.

A preferred method for attaching antibodies or other proteins to a solid-state substrate is to functionalize the substrate with an amino- or thiol-silane, and then to activate the functionalized substrate with a homobifunctional cross-linker agent such as (Bis-sulfo-succinimidyl suberate (BS^3) or a heterobifunctional cross-linker agent such as GMBS. For cross-linking with GMBS, glass substrates are chemically functionalized by immersing in a solution of mercaptopropyltrimethoxysilane (1% vol/vol in 95% ethanol pH 5.5) for 1 hour, rinsing in 95% ethanol and heating at 120°C for 4 hrs. Thiol-derivatized slides are activated by immersing in a 0.5 mg/ml solution of GMBS in 1% dimethylformamide, 99% ethanol for 1 hour at room temperature. Antibodies or proteins are added directly to the activated substrate, which are then blocked with solutions containing agents such as 2% bovine serum albumin, and air-dried. Other standard immobilization chemistries are known by those of skill in the art.

Each of the components (analyte capture agents, accessory molecules, and/or analytes) immobilized on the solid support preferably is located in a different predefined region of the solid support. The different locations preferably are different reaction chambers. Each of the different predefined regions can be physically

separated from each other of the different regions. The distance between the different predefined regions of the solid support can be either fixed or variable. For example, in an array, each of the components can be arranged at fixed distances from each other, while components associated with beads will not be in a fixed spatial relationship. In particular, the use of multiple solid support units (for example, multiple beads) will result in variable distances.

Components can be associated or immobilized on a solid support at any density. Components preferably are immobilized to the solid support at a density exceeding 400 different components per cubic centimeter. Arrays of components can have any number of components. For example, an array can have at least 1,000 different components immobilized on the solid support, at least 10,000 different components immobilized on the solid support, at least 100,000 different components immobilized on the solid support, or at least 1,000,000 different components immobilized on the solid support.

L. DNA polymerases

DNA polymerases useful in the disclosed method must be capable, either alone or in combination with a compatible strand displacement factor, perform rolling circle replication of primed single-stranded circles. Such polymerases are referred to herein as rolling circle DNA polymerases. It is preferred that a rolling circle DNA polymerase lack a 5' to 3' exonuclease activity. Strand displacement is necessary to result in synthesis of multiple tandem copies of an amplification target circle. A 5' to 3' exonuclease activity, if present, might result in the destruction of the synthesized strand. It is also preferred that DNA polymerases for use in the disclosed method are highly processive. The suitability of a DNA polymerase for use in the disclosed method can be readily determined by assessing its ability to carry out strand displacement replication. Preferred strand displacement DNA polymerases are Bst large fragment DNA polymerase (Exo(-) Bst; Aliotta *et al.*, *Genet. Anal. (Netherlands)* **12**:185-195 (1996)), exo(-)Bca DNA polymerase (Walker and Linn, *Clinical Chemistry* **42**:1604-1608 (1996)), and bacteriophage ϕ29 DNA polymerase (U.S. Patent Nos. 5,198,543 and 5,001,050 to Blanco *et al.*). Other useful polymerases include phage M2 DNA polymerase (Matsumoto *et al.*, *Gene* **84**:247 (1989)), phage ϕPRD1 DNA polymerase (Jung *et al.*, *Proc. Natl. Acad. Sci. USA* **84**:8287 (1987)), exo(-)VENT®

DNA polymerase (Kong *et al.*, *J. Biol. Chem.* **268**:1965-1975 (1993)), Klenow fragment of DNA polymerase I (Jacobsen *et al.*, *Eur. J. Biochem.* **45**:623-627 (1974)), T5 DNA polymerase (Chatterjee *et al.*, *Gene* **97**:13-19 (1991)), Sequenase (U.S. Biochemicals), PRD1 DNA polymerase (Zhu and Ito, *Biochim. Biophys. Acta.* **1219**:267-276 (1994)), and T4 DNA polymerase holoenzyme (Kaboord and Benkovic, *Curr. Biol.* **5**:149-157 (1995)). Bst DNA polymerase is most preferred.

Strand displacement can be facilitated through the use of a strand displacement factor, such as helicase. It is considered that any DNA polymerase that can perform strand displacement replication in the presence of a strand displacement factor is suitable for use in the disclosed method, even if the DNA polymerase does not perform strand displacement replication in the absence of such a factor. Strand displacement factors useful in strand displacement replication include BMRF1 polymerase accessory subunit (Tsurumi *et al.*, *J. Virology* **67**(12):7648-7653 (1993)), adenovirus DNA-binding protein (Zijderveld and van der Vliet, *J. Virology* **68**(2):1158-1164 (1994)), herpes simplex viral protein ICP8 (Boehmer and Lehman, *J. Virology* **67**(2):711-715 (1993); Skaliter and Lehman, *Proc. Natl. Acad. Sci. USA* **91**(22):10665-10669 (1994)); single-stranded DNA binding proteins (SSB; Rigler and Romano, *J. Biol. Chem.* **270**:8910-8919 (1995)); phage T4 gene 32 protein (Villemain and Giedroc, *Biochemistry* **35**:14395-14404 (1996); and calf thymus helicase (Siegel *et al.*, *J. Biol. Chem.* **267**:13629-13635 (1992)).

The ability of a polymerase to carry out rolling circle replication can be determined by using the polymerase in a rolling circle replication assay such as those described in Fire and Xu, *Proc. Natl. Acad. Sci. USA* 92:4641-4645 (1995).

The materials described above can be packaged together in any suitable combination as a kit useful for performing the disclosed method. For example, a kit can include a plurality of reporter binding molecules and/or a plurality of analyte capture agents. The analyte capture agents in the kit can be associated with a solid support.

Method

The disclosed method is a form of rolling circle amplification (RCA) where a reporter binding molecule provides the amplification target circle for amplification. The disclosed method allows RCA to produce an amplified signal (that is, tandem

sequence DNA (TS-DNA)) based on association of the reporter binding molecule with a target molecule (also referred to as an analyte). The specific amplification target circle that is a part of the reporter binding molecule provides the link between the specific interaction of the reporter binding molecule to an analyte (via the affinity portion of the reporter binding molecule) and RCA. Once the reporter binding molecule is associated with an analyte, a rolling circle replication primer is hybridized to the amplification target circle (ATC) of the reporter binding molecule, followed by amplification of the ATC by RCA (a secondary DNA strand displacement primer is also used if exponential RCA is performed). The disclosed method can be performed using any analyte. Preferred analytes are proteins, peptides, nucleic acids, including amplified nucleic acids such as TS-DNA and amplification target circles, antigens and ligands. Target molecules for the disclosed method are generally referred to herein as analytes.

The amplification target circle is released from the reporter binding molecule prior to amplification. Such release, referred to herein as decoupling, can be accomplished in any suitable manner. In general, the manner in which the amplification target circle is associated with, or linked or coupled to, the reporter binding molecule determines the form of decoupling. For example, where the amplification target circle is base paired to a circle capture probe in the reporter binding molecule, the amplification target circle can be decoupled from the reporter binding molecule by disrupting the base pairing. Where the amplification target circle is covalently coupled to the reporter binding molecule via circle linker having a cleavable bond, the amplification target circle can be decoupled from the reporter binding molecule by cleaving the cleavable bond. To identify analytes using the amplification target circles, reporter binding molecules that are not associated with analytes should be removed prior to decoupling.

Following decoupling, the amplification target circle can be replicated by rolling circle amplification. If multiple different analytes are to be detected, the amplification products of amplification target circles associated with different analytes should be distinguishable. This can be accomplished in any suitable manner. For example, the amplification target circles can be in separate locations prior to decoupling and remain separated following decoupling. The separate locations could

be determined, for example, by the location of the analytes with which the amplification target circles are associated. In this case, some or all of the amplification target circles can be the same (thus producing the same amplification product). The different locations of the amplification products identifies the analyte involved. As another example, some or all of the amplification target circles that are associated with different analytes can be different (thus producing different amplification products). The different amplification products identify the analytes involved. Even if the amplification target circles are mixed together and/or amplified in the same reaction, the different amplification target circles (and thus the different corresponding analytes) can be detected and distinguished based on the differences in the amplification products.

The amplification products of RCA can be detected using any suitable technique. Real time detection, that is, detection during the RCA reaction is a preferred mode of detection with the disclosed method. Real time detection can be facilitated by use of Amplifluor™ primers. Amplifluor™ primers produce a fluorescent signal when they become incorporated into a replicated strand and are based paired with a complementary strand.

The disclosed method is particularly useful for generating a profile of analytes present in a given sample. For example, the presence and amount of various proteins present in cells can be assessed, thus providing a direct protein expression profile. Such analysis, a form of proteomics, is analogous to genomics analysis of the presence and expression of nucleic acids. Multiple analyte analysis, such as the proteomics mode of the disclosed invention, is preferably carried out using sets of analyte capture agents. By including in the set analyte capture agents specific for all of the analytes to be assessed, the full range of analytes can be assayed in a single procedure. This form of the method also allows easy comparison of the same suite of analytes in multiple samples.

In a preferred form of the disclosed method, the analytes in two (or more) different samples can be assessed in the same reaction by mixing a different set of reporter binding molecules with each sample. Each set of reporter binding molecules has the same set of specific binding molecules but a different set of amplification target circles. By making the different amplification target circles specific for different

rolling circle replication primers (and different secondary DNA strand displacement primers if exponential RCA is performed), the amplification of a specific amplification target circle will indicate in which sample the corresponding analyte is present. Alternatively, by using different detection tag sequences in the different amplification target circles the amplification products of the different amplification target circles can be distinguished. This allows the identification of the analyte corresponding to a given amplification target circle.

Identification of multiple analytes can be facilitated by using analyte capture agents to capture and/or separate analytes based on their identity. For example, a set of immobilized analyte capture agents can be used to associate particular analytes with predefined regions on a solid support. Detection of an analyte in that region identifies the analyte. One useful form of analyte capture agent is peptides. When various peptides are immobilized on a solid support, they can be used as "bait" for analytes. For example, an array of different peptides can be used to access whether a sample has analytes that interact with any of the peptides. Comparisons of different samples can be made by, for example, noting differences in the peptides to which analytes in the different samples become associated. In another form of the disclosed method, a set of analyte capture agents specific for analytes of interest can be used to access the presence of a whole suite of analytes in a sample.

In another form of the disclosed method, accessory molecules can be used to affect the interaction of analytes with specific binding molecules or analyte capture agents. For example, the disclosed method can be used to screen for competitors (or binding co-factors) of an analyte interaction with a specific binding molecule or analyte capture agent. If an accessory molecule affects interaction of the analyte, the results of RCA will change since the association of the reporter binding molecule to the analyte (or of the analyte capture agent to the analyte) will be lost or gained.

Different modified forms of analytes can also be detected with the disclosed method. For example, phosphorylated and glycosylated forms of proteins can be detected. This can be accomplished, for example, by using reporter binding molecules having specific binding molecules specific for the different forms of analyte.

In another aspect, the disclosed method involves immobilization of analytes present in complex biological samples and determining and quantitating their presence

in the samples. In another aspect, the disclosed method involves multiplexed detection and quantitation of more than one analytes in a sample. For example, a solid support containing immobilized capture antibodies can be incubated with sample containing a mixture of protein analytes to be detected. The solid support next can be incubated with a mixture containing at least one biotinylated antibody for each analyte. An immunoRCA assay then can be employed for detection and quantitation.

In another aspect, an immunoRCA assay can be performed in 16 microwell-glass slides, wherein each well is separated by a Teflon mask. Each of these wells can be used, for example, to assay different samples and controls, to assay different analytes, or to assay different sets of analytes. Multiwell slides also can be printed with arrays of anti-IgE capture antibodies in the wells. Semi-automation of immunoRCA assays on allergen microarrays in this multiwell format can be implemented, for example, on an inexpensive Beckman BioMek liquid handling robot.

ImmunoRCA assay can be applied to other multiplexed antibody assays. For example, certain immunological reactions are caused by specific IgG_4 rather than IgE (AAAI Board of Directors, *J Allergy Clin Immunol.* 95:652-654 (1995)). The use of an anti-human IgG_4 conjugated to a DNA circle that is different in sequence from the DNA circle conjugated to an anti-IgE would allow the simultaneous measurement of allergen-specific IgG_4 and IgE. Such an assay can be used during allergen desensitization therapy or for monitoring response to anti-IgE therapy (Chang *Nature Biotech.* 18:157-162 (2000)).

The disclosed method generally includes the following steps:

(a) Bringing into contact one or more analyte samples and one or more reporter binding molecules, incubating the analyte samples and the reporter binding molecules under conditions that promote interaction of the specific binding molecules and analytes, and separating the specific binding molecules that interact with the analytes from the specific binding molecules that do not interact with the analytes. Each reporter binding molecule comprises a specific binding molecule and an amplification target circle, wherein each specific binding molecule interacts with an analyte directly or indirectly.

(b) Decoupling the amplification target circles from the reporter binding molecules that interact with the analytes.

(c) Bringing into contact the amplification target circles and one or more rolling circle replication primers, and incubating the rolling circle replication primers and amplification target circles under conditions that promote hybridization between the amplification target circles and the rolling circle replication primers. The amplification target circles each comprise a single-stranded, circular DNA molecule comprising a primer complement portion, wherein the primer complement portion is complementary to at least one of the rolling circle replication primers.

(d) Incubating the rolling circle replication primers and amplification target circles under conditions that promote replication of the amplification target circles. Replication of the amplification target circles results in the formation of tandem sequence DNA, wherein detection of tandem sequence DNA indicates the presence of the corresponding analytes.

The method can also be performed where at least one of the reporter binding molecules further comprises a circle capture probe, and where the amplification target circle of the reporter binding molecule is associated with the reporter binding molecule via a non-covalent interaction with the circle capture probe. The non-covalent interaction can be base pairing. Decoupling of the amplification target circle can be accomplished by disrupting the base pairing. Base pairing can be disrupted by heating the reporter binding molecules. The circle capture probe can comprise an oligonucleotide. In some embodiments, the oligonucleotide cannot be extended. For example, the oligonucleotide can comprise a 3' end and a 5' end, wherein only the 5' end is free. The oligonucleotide can be coupled to the specific binding molecule of the reporter binding molecule via the 3' end of the oligonucleotide, the 3' end of the oligonucleotide can be blocked, or the oligonucleotide can be blocked.

The method can also be performed where at least one of the reporter binding molecules further comprises a circle linker, and where the amplification target circle of the reporter binding molecule is coupled to the reporter binding molecule via the circle linker. The circle linker can comprise a cleavable bond. Decoupling of the amplification target circle can be accomplished by cleaving the cleavable bond. In some embodiments, the cleavable bond can be cleaved by treatment with a reducing agent. The cleavable bond can be a disulfide bond. For example the circle linker can comprise dithiobis succinimidyl propionate, dimethyl 3,3'-dithiobispropionimidate,

dithio-bis-maleimidoethane, 3,3'-dithiobis sulfosuccinimidyl propionate, succinimidyl 6-[3-(2-pyridyldithio)-propionamido]hexonate, or N-succinimidyl 3-[2-pyridyldithio]propionate. In some embodiments, the cleavable bond can be cleaved by treatment with periodate. The cleavable bond can be a dihydroxy bond. For example, the circle linker can comprise 1,4 bis-maleimidyl-2,3-dihydroxybutane, disuccinimidyl tartrate, or disulfosuccinimidyl tartrate. The circle linker can be coupled to the amplification target circle via a reactive group on the amplification target circle. The reactive group can be an allyl amino group.

The method can be performed wherein a plurality of reporter binding molecules are brought into contact with the one or more analyte samples; wherein a plurality of analyte samples are brought into contact with the one or more reporter binding molecules; wherein at least one of the analytes is a protein or peptide; wherein at least one of the analytes is a lipid, glycolipid, or proteoglycan; wherein at least one of the analytes is from a human source; wherein at least one of the analytes is from a non-human source; wherein none of the analytes are nucleic acids; wherein at least one of the specific binding molecules is an antibody specific for at least one of the analytes; wherein at least one of the specific binding molecules is a molecule that specifically binds to at least one of the analytes; wherein at least one of the specific binding molecules is a molecule that specifically binds to at least one of the analytes in combination with an accessory molecule; and/or wherein the specific binding molecules and analytes interact by binding to each other directly or indirectly. The reporter binding molecules can be at least 20% pure, at least 50% pure, at least 80% pure, or at least 90% pure.

The method can also include bringing into contact at least one of the analyte samples and one or more analyte capture agents, and separating analyte capture agents from the analyte samples, thus separating analytes from the analyte samples. Each analyte capture agent interacts with an analyte directly or indirectly, and at least one analyte, if present in the analyte sample, interacts with at least one analyte capture agent. The method can also include bringing into contact at least one of the analyte samples and at least one of the reporter binding molecules with at least one accessory molecule. The accessory molecule affects the interaction of at least one of the analytes

and at least one of the specific binding molecules or at least one of the analyte capture agents.

The method can further comprise, simultaneous with, or following, step (d), bringing into contact a secondary DNA strand displacement primer and the tandem sequence DNA, and incubating under conditions that promote (i) hybridization between the tandem sequence DNA and the secondary DNA strand displacement primer, and (ii) replication of the tandem sequence DNA, wherein replication of the tandem sequence DNA results in the formation of secondary tandem sequence DNA. In this form of the method, the rolling circle replication primer can hybridize to the secondary tandem sequence DNA and the secondary tandem sequence DNA can be replicated to form tertiary tandem sequence DNA. The rolling circle replication primer and secondary DNA strand displacement primer can continue to hybridize with and replicate the tandem sequence DNA, secondary tandem sequence DNA, tertiary tandem sequence DNA (and other higher order tandem sequence DNAs) to form more amplified DNA (that is, various generations of tandem sequence DNA).

This form of the method can further comprise, simultaneous with, or following, step (d), bringing into contact a tertiary DNA strand displacement primer and the secondary tandem sequence DNA, and incubating under conditions that promote (i) hybridization between the secondary tandem sequence DNA and the tertiary DNA strand displacement primer, and (ii) replication of the secondary tandem sequence DNA, wherein replication of the secondary tandem sequence DNA results in the formation of tertiary tandem sequence DNA. The tertiary DNA strand displacement primer and secondary DNA strand displacement primer can continue to hybridize with and replicate the tandem sequence DNA, secondary tandem sequence DNA, tertiary tandem sequence DNA (and other higher order tandem sequence DNAs) to form more amplified DNA (that is, various generations of tandem sequence DNA). In this form of the method, the rolling circle replication primer can be used as the tertiary DNA strand displacement primer.

The method can be performed wherein a plurality of reporter binding molecules are brought into contact with one or more analyte samples, wherein two or more of the amplification target circles are replicated in the same reaction, wherein the amplification target circles replicated in the same reaction are different, wherein each

different amplification target circle produces a different tandem sequence DNA, wherein the presence or absence of different analytes is indicated by the presence or absence of corresponding tandem sequence DNA. Replication of each different amplification target circle can be primed by a different one of the rolling circle replication primers.

The method can be performed wherein at least one of the analytes is associated with a solid support. The solid support can comprise one or more reaction chambers, wherein a plurality of the analytes associated with the solid support are associated with the solid support in the same reaction chamber. At least one of the analytes associated with the solid support can be associated with the solid support indirectly. The analytes associated with the solid support can interact with analyte capture agents, wherein the analyte capture agents are associated with the solid support thereby indirectly associating the analytes with the solid support.

The method can be performed wherein at least one specific binding molecule interacts with at least one analyte indirectly. The analyte can interact with an analyte capture agent, wherein the specific binding molecule interacts with the analyte capture agent thereby indirectly associating the specific binding molecule with the analyte. The method can be performed wherein at least one of the analytes is a modified form of another analyte, wherein the specific binding molecule of at least one of the reporter binding molecules interacts, directly or indirectly, with the analyte that is a modified form of the other analyte, wherein the specific binding molecule of another reporter binding molecule interacts, directly or indirectly, with the other analyte. The analytes can be proteins, wherein the modification of the modified form of the other analyte can be a post-translational modification. The modification can be phosphorylation or glycosylation.

The method can be performed wherein detection of the tandem sequence DNA is accomplished by mixing a set of detection probes with the tandem sequence DNA under conditions that promote hybridization between the tandem sequence DNA and the detection probes. A plurality of different tandem sequence DNAs can be detected separately and simultaneously via multiplex detection. The set of detection probes can be labeled using combinatorial multicolor coding.

In one form of the method, the specific binding molecules that interact with the analytes can be separated by bringing into contact at least one of the analyte samples and one or more analyte capture agents, and separating analyte capture agents from the analyte samples, thus separating specific binding molecules that interact with the analytes from the analyte samples. Each analyte capture agent can interact with an analyte directly or indirectly, and at least one analyte, if present in the analyte sample, can interact with at least one analyte capture agent. At least one analyte capture agent can be associated with a solid support, wherein analytes that interact with the analyte capture agent associated with a solid support become associated with the solid support. The solid support can comprise one or more reaction chambers, wherein a plurality of the analyte capture agents are located in the same reaction chamber on the solid support.

In this form of the method, a plurality of reporter binding molecules can be brought into contact with one or more analyte samples, wherein two or more of the amplification target circles are replicated in the same reaction chamber of the solid support, wherein the amplification target circles replicated in the same reaction chamber of the solid support are different, and wherein each different amplification target circle produces a different tandem sequence DNA. The presence or absence of different analytes is indicated by the presence or absence of corresponding tandem sequence DNA. Replication of each different amplification target circle can be primed by a different one of the rolling circle replication primers.

The solid support can comprise acrylamide, agarose, cellulose, cellulose, nitrocellulose, glass, gold, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, glass, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, functionalized silane, polypropylfumerate, collagen, glycosaminoglycans, or polyamino acids.

This form of the method can further comprise bringing into contact at least one of the analyte samples and at least one of the reporter binding molecules with at least one accessory molecule, wherein the accessory molecule affects the interaction of at least one of the analytes and at least one of the specific binding molecules or at least one of the analyte capture agents. The accessory molecule can be brought into contact

with at least one of the analyte samples, at least one of the reporter binding molecules, or both, prior to, simultaneous with, or following step (a). At least one analyte capture agent can be associated with a solid support, wherein the accessory molecule is associated with the solid support. The accessory molecule can be associated with the solid support by bringing the accessory molecule into contact with the solid support prior to, simultaneous with, or following step (a). The accessory molecule can be a protein kinase, a protein phosphatase, an enzyme, or a compound. The accessory molecule can be a molecule of interest, wherein one or more of the analytes are test molecules, wherein interactions of the test molecules with the molecule of interest are detected. At least one of the analytes can be a molecule of interest, wherein the accessory molecule is a test molecule, wherein interactions of the test molecule with the molecule of interest are detected.

In this form of the method, the analyte samples can include one or more first analyte samples and one or more second analyte samples, wherein the reporter binding molecules include one or more first reporter binding molecules and one or more second reporter binding molecules. The method can further comprise, following step (a) and prior to bringing the analyte samples and the solid support into contact, mixing one or more of the first analyte samples and one or more of the second analyte samples. For each first reporter binding molecule there is a matching second reporter binding molecule, wherein the specific binding molecules of the first reporter binding molecules interacts with the same analyte as the specific binding molecules of the matching second reporter binding molecule. The amplification target circle of each different reporter binding molecule is different, and each different amplification target circle produces a different tandem sequence DNA. The presence or absence of the same analyte in different analyte samples is indicated by the presence or absence of corresponding tandem sequence DNA. Replication of each different amplification target circle can be primed by a different one of the rolling circle replication primers. The tandem sequence DNA corresponding to one of the analytes and produced in association with a first reporter binding molecule is in the same location on the solid support as tandem sequence DNA corresponding to the same analyte and produced in association with the matching second reporter binding molecule. The presence or

absence of the same analyte in different analyte samples is indicated by the presence or absence of corresponding tandem sequence DNA.

In this form of the method, at least one of the analyte capture agents is a molecule of interest, wherein one or more of the analytes are test molecules, wherein interactions of the test molecules with the molecule of interest are detected; or at least one of the analytes is a molecule of interest, wherein one or more of the analyte capture agents are test molecules, wherein interactions of the test molecules with the molecule of interest are detected.

Another form of the method further comprises, prior to, simultaneous with, or following step (a), bringing into contact one or more first analyte capture agents and one or more first analyte samples, and bringing into contact one or more second analyte capture agents and one or more second analyte samples. Each analyte capture agent comprises an analyte interaction portion and a capture portion, wherein for each first analyte capture agent there is a matching second analyte capture agent. The analyte interaction portions of the first analyte capture agents interact with the same analyte as the analyte interaction portions of the matching second analyte capture agents. The capture portions of the first and second analyte capture agents each interact with a specific binding molecule of one or more of the reporter binding molecules, wherein the capture portions of the first analyte capture agents interact with different specific binding molecules than the capture portions of the matching second analyte capture agents. Each different specific binding molecule is part of a different one of the reporter binding molecules, wherein the amplification target circle of each different reporter binding molecule is different, wherein replication of each different amplification target circle is primed by a different one of the rolling circle replication primers, wherein each different amplification target circle produces a different tandem sequence DNA, wherein the amplification target circle of a reporter binding molecule that comprises a specific binding molecule that interacts with an analyte capture agent corresponds to the analyte capture agent. The presence or absence of the same analyte in different analyte samples is indicated by the presence or absence of corresponding tandem sequence DNA.

This form of the method can further comprise mixing one or more of the first analyte samples and one or more of the second analyte samples, or mixing the one or

more first analyte capture agents and the one or more second analyte capture agents. Mixing the one or more first analyte capture agents and the one or more second analyte capture agents can be accomplished by associating, simultaneously or sequentially, the one or more first analyte capture agents and the one or more second analyte capture agents with the same solid support.

In this form of the method, the tandem sequence DNA corresponding to one of the analytes and produced in association with a first analyte capture agent can be in the same location as, and can be simultaneously detected with, tandem sequence DNA corresponding to the same analyte and produced in association with the matching second analyte capture agent. The presence or absence of the same analyte in different analyte samples is indicated by the presence or absence of corresponding tandem sequence DNA.

In this form of the method, the capture portion of each first analyte capture agent can be the same, wherein the reporter binding molecules corresponding to the first analyte capture agents are the same, wherein the amplification target circles corresponding to the first analyte capture agents are the same. The capture portion of each second analyte capture agent can be the same, wherein the reporter binding molecules corresponding to the second analyte capture agents are the same, wherein the amplification target circles corresponding to the second analyte capture agents are the same.

In another form of the method, at least one accessory molecule can be brought into contact with at least one of the analyte samples and at least one of the reporter binding molecules, wherein the accessory molecule affects the interaction of at least one of the analytes and at least one of the specific binding molecules or at least one of the analyte capture agents. The accessory molecule can compete with the interaction of at least one of the specific binding molecules or at least one of the analyte capture agents. The accessory molecule can be an analog of at least one of the analytes. The accessory molecule can facilitate the interaction of at least one of the specific binding molecules or at least one of the analyte capture agents. The accessory molecule can be brought into contact with at least one of the analyte samples, at least one of the reporter binding molecules, or both, prior to, simultaneous with, or following step (a).

In this form of the method, the accessory molecule can be a protein kinase, a protein phosphatase, an enzyme, or a compound. The accessory molecule can be at least 20% pure, at least 50% pure, at least 80% pure, or at least 90% pure.

Another form of the disclosed method generally includes the following steps:

(a) Bringing into contact one or more analyte samples and one or more analyte capture agents, and incubating the analyte samples and the analyte capture agents under conditions that promote interaction of the analyte capture agents and analytes. Each analyte capture agent can interact with an analyte directly or indirectly. At least one analyte, if present in the analyte sample, can interact with at least one analyte capture agent.

(b) Bringing into contact at least one of the analyte samples and one or more reporter binding molecules, incubating the analyte samples and the reporter binding molecules under conditions that promote interaction of the specific binding molecules and analyte capture agents, and separating the specific binding molecules that interact with the analyte capture agents from the specific binding molecules that do not interact with the analyte capture agents. Each reporter binding molecule can comprise a specific binding molecule and an amplification target circle, and each specific binding molecule can interact with an analyte capture agent directly or indirectly.

(c) Decoupling the amplification target circles from the reporter binding molecules that interact with the analyte capture agents.

(d) Bringing into contact the amplification target circles and one or more rolling circle replication primers, and incubating the rolling circle replication primers and amplification target circles under conditions that promote hybridization between the amplification target circles and the rolling circle replication primers. The amplification target circles each can comprise a single-stranded, circular DNA molecule comprising a primer complement portion, and the primer complement portion is complementary to at least one of the rolling circle replication primers.

(e) Incubating the rolling circle replication primers and amplification target circles under conditions that promote replication of the amplification target circles. Replication of the amplification target circles results in the formation of tandem sequence DNA, wherein detection of tandem sequence DNA indicates the presence of the corresponding analytes.

Another form of the disclosed method generally includes the following steps:

(a) Treating one or more analyte samples so that one or more analytes are modified.

(b) Bringing into contact at least one of the analyte samples and one or more reporter binding molecules, incubating the analyte samples and the reporter binding molecules under conditions that promote interaction of the specific binding molecules and modified analytes, and separating the specific binding molecules that interact with the modified analytes from the specific binding molecules that do not interact with the modified analytes. Each reporter binding molecule can comprise a specific binding molecule and an amplification target circle, and each specific binding molecule can interact with a modified analyte directly or indirectly.

(c) Decoupling the amplification target circles from the reporter binding molecules that interact with the modified analytes.

(d) Bringing into contact the amplification target circles and one or more rolling circle replication primers, and incubating the rolling circle replication primers and amplification target circles under conditions that promote hybridization between the amplification target circles and the rolling circle replication primers. The amplification target circles each can comprise a single-stranded, circular DNA molecule comprising a primer complement portion, and the primer complement portion is complementary to at least one of the rolling circle replication primers.

(e) Incubating the rolling circle replication primers and amplification target circles under conditions that promote replication of the amplification target circles. Replication of the amplification target circles results in the formation of tandem sequence DNA, wherein detection of tandem sequence DNA indicates the presence of the corresponding modified analytes.

In this form of the method, all of the analytes can be modified by associating a modifying group to the analytes, wherein the modifying group is the same for all of the analytes, wherein all of the specific binding molecules interact with the modifying group.

Another form of the disclosed method generally includes the following steps:

(a) Bringing into contact one or more analyte samples and a set of analyte capture agents, a set of accessory molecules, or both. Each analyte capture agent can interact with an analyte directly or indirectly.

(b) Prior to, simultaneous with, or following step (a), bringing into contact at least one of the analyte samples and one or more reporter binding molecules. Each reporter binding molecule can comprise a specific binding molecule and an amplification target circle, each specific binding molecule can interact with an analyte directly or indirectly, and each accessory molecule can affect the interaction of at least one of the analytes and at least one of the specific binding molecules or at least one of the analyte capture agents.

(c) Simultaneous with, or following, steps (a) and (b), incubating the analyte samples, the analyte capture agents, the accessory molecules, and the reporter binding molecules under conditions that promote interaction of the specific binding molecules, analytes, analyte capture agents, and accessory molecules, separating the specific binding molecules that interact with the analytes from the specific binding molecules that do not interact with the analytes, and decoupling the amplification target circles from the reporter binding molecules that interact with the analytes.

(d) Bringing into contact the amplification target circles and one or more rolling circle replication primers, and incubating the rolling circle replication primers and amplification target circles under conditions that promote hybridization between the amplification target circles and the rolling circle replication primers. The amplification target circles each can comprise a single-stranded, circular DNA molecule comprising a primer complement portion, and the primer complement portion is complementary to at least one of the rolling circle replication primers.

(e) Incubating the reporter binding molecules and amplification target circles under conditions that promote replication of the amplification target circles. Replication of the amplification target circles results in the formation of tandem sequence DNA, wherein detection of tandem sequence DNA indicates the presence of the corresponding analytes.

The method can also be performed where the analyte capture agents are immobilized on a solid support, where the solid support comprises one or more reaction

chambers, and where a plurality of the analyte capture agents are immobilized in the same reaction chamber of the solid support. The analyte capture agents can be immobilized to the solid support at a density exceeding 400 different analyte capture agents per cubic centimeter. The analyte capture agents can be peptides. Each of the different peptides can be at least 4 amino acids in length, from about 4 to about 20 amino acids in length, at least 10 amino acids in length, or at least 20 amino acids in length.

The solid support can comprise a plurality of reaction chambers. The solid support can comprise acrylamide, agarose, cellulose, cellulose, nitrocellulose, glass, gold, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, glass, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, functionalized silane, polypropylfumerate, collagen, glycosaminoglycans, or polyamino acids. The analyte capture agents in the reaction chambers can be at least 20% pure, at least 50% pure, at least 80% pure, or at least 90% pure.

Another form of the disclosed method generally includes:

Bringing into contact one or more analyte samples and one or more reporter binding molecules. Each reporter binding molecule can comprise a specific binding molecule and an amplification target circle, and each specific binding molecule can interact with an analyte directly or indirectly.

Separating the specific binding molecules that interact with the analytes from the specific binding molecules that do not interact with the analytes.

Decoupling the amplification target circles from the reporter binding molecules that interact with the analytes.

Replicating the amplification target circles. Replication of the amplification target circles results in the formation of tandem sequence DNA, wherein detection of tandem sequence DNA indicates the presence of the corresponding analytes.

Another form of the disclosed method generally includes:

Bringing into contact one or more analyte samples and one or more analyte capture agents. Each analyte capture agents can interact with an analyte directly or indirectly.

Bringing into contact at least one of the analyte samples and one or more reporter binding molecules. Each reporter binding molecule can comprise a specific binding molecule and an amplification target circle, and each specific binding molecule can interact with an analyte capture agent directly or indirectly.

Separating the specific binding molecules that interact with the analyte capture agents from the specific binding molecules that do not interact with the analyte capture agents.

Decoupling the amplification target circles from the reporter binding molecules that interact with the analyte capture agents.

Replicating the amplification target circles. Replication of the amplification target circles results in the formation of tandem sequence DNA, wherein detection of tandem sequence DNA indicates the presence of the corresponding analytes.

Another form of the disclosed method generally includes:

Treating one or more analyte samples so that one or more analytes are modified.

Bringing into contact at least one analyte samples and one or more reporter binding molecules. Each reporter binding molecule can comprise a specific binding molecule and an amplification target circle, and each specific binding molecule can interact with a modified analyte directly or indirectly.

Separating the specific binding molecules that interact with the modified analytes from the specific binding molecules that do not interact with the modified analytes.

Decoupling the amplification target circles from the reporter binding molecules that interact with the modified analytes.

Replicating the amplification target circles. Replication of the amplification target circles results in the formation of tandem sequence DNA, wherein detection of tandem sequence DNA indicates the presence of the corresponding modified analytes.

Another form of the disclosed method generally includes:

Bringing into contact one or more analyte samples and a set of analyte capture agents, a set of accessory molecules, or both, wherein each analyte capture agent can interact with an analyte directly or indirectly,

Bringing into contact at least one of the analyte samples and one or more reporter binding molecules. Each reporter binding molecule can comprise a specific

binding molecule and an amplification target circle, each specific binding molecule can interact with an analyte directly or indirectly, and each accessory molecule can affect the interaction of at least one of the analytes and at least one of the specific binding molecules or at least one of the analyte capture agents.

Separating the specific binding molecules that interact with the analytes from the specific binding molecules that do not interact with the analytes.

Decoupling the amplification target circles from the reporter binding molecules that interact with the analytes.

Replicating the amplification target circles. Replication of the amplification target circles results in the formation of tandem sequence DNA, wherein detection of tandem sequence DNA indicates the presence of the corresponding analytes.

The amplification target circles serve as substrates for a rolling circle replication. This reaction requires the addition of two reagents: (a) a rolling circle replication primer, which is complementary to the primer complement portion of the ATC, and (b) a rolling circle DNA polymerase. The DNA polymerase catalyzes primer extension and strand displacement in a processive rolling circle polymerization reaction that proceeds as long as desired, generating a molecule of up to 100,000 nucleotides or larger that contains up to approximately 1000 tandem copies of a sequence complementary to the amplification target circle. A preferred rolling circle DNA polymerase is Bst DNA polymerase.

Many different forms of RCA can be used in the disclosed method, most of which are described in U.S. Patent No. 5,854,033 and WO 97/19193. For example, linear rolling circle amplification (LRCA) involves the basic rolling circle replication of an amplification target circle to form a strand of TS-DNA. Exponential rolling circle amplification (ERCA) involves replication of TS-DNA by strand displacement replication initiated at the numerous repeated sequences in the TS-DNA. Multiple priming on both strands of TS-DNA leads to an exponential amplification of sequences in the amplification target circle. ERCA is preferred for the disclosed method. If desired, the TS-DNA can be collapsed into a compact structure for detection as described in WO 97/19193.

During rolling circle replication one may additionally include radioactive or modified nucleotides such as bromodeoxyuridine triphosphate, in order to label the

DNA generated in the reaction. Alternatively, one may include suitable precursors that provide a binding moiety such as biotinylated nucleotides (Langer *et al.* (1981)).

Examples of proteins that can be analyzed and detected using the disclosed method include IL-1alpha, IL-1beta, IL-1RA, IL-2, IL-3, IL-4, IL-6, IL-6R, IL-7, IL-8, IL-9, IL-10, GROalpha, MIP-1alpha, MIP-1beta, MCP, RANTES, MIF, G-CSF, GM-CSF, M-CSF, EGF, FGF acidic, FGF basic, IGF-1, IGF-2, IFN-gamma, TGF-beta, TNF-alpha, TNF-beta, TNF-RI, TNF-RII, ICAM-1, ICAM-2, IL-2Ra, IL-4R, IL-5, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IP-10, FGF-4, FGF-6, MCP-2, and MCP-3.

A. Detection of Amplification Products

Amplification products can be detected directly by, for example, primary labeling or secondary labeling, as described below.

i. Primary Labeling

Primary labeling consists of incorporating labeled moieties, such as fluorescent nucleotides, biotinylated nucleotides, digoxygenin-containing nucleotides, or bromodeoxyuridine, during strand displacement replication. For example, one may incorporate cyanine dye deoxyuridine analogs (Yu *et al.*, *Nucleic Acids Res.*, **22**:3226-3232 (1994)) at a frequency of 4 analogs for every 100 nucleotides. A preferred method for detecting nucleic acid amplified *in situ* is to label the DNA during amplification with BrdUrd, followed by binding of the incorporated BrdU with a biotinylated anti-BrdU antibody (Zymed Labs, San Francisco, CA), followed by binding of the biotin moieties with Streptavidin-Peroxidase (Life Sciences, Inc.), and finally development of fluorescence with Fluorescein-tyramide (DuPont de Nemours & Co., Medical Products Dept.). Other methods for detecting nucleic acid amplified *in situ* include labeling the DNA during amplification with 5-methylcytosine, followed by binding of the incorporated 5-methylcytosine with an antibody (Sano *et al., Biochim. Biophys. Acta* **951**:157-165 (1988)), or labeling the DNA during amplification with aminoallyl-deoxyuridine, followed by binding of the incorporated aminoallyl-deoxyuridine with an Oregon Green® dye (Molecular Probes, Eugene, OR) (Henegariu *et al., Nature Biotechnology* **18**:345-348 (2000)).

Another method of labeling amplified nucleic acids is to incorporate 5-(3-aminoallyl)-dUTP (AAdUTP) in the nucleic acid during amplification followed by

chemical labeling at the incorporated nucleotides. Incorporated 5-(3-aminoallyl)-deoxyuridine (AAdU) can be coupled to labels that have reactive groups that are capable of reacting with amine groups. AAdUTP can be prepared according to Langer et al. (1981). Proc. Natl. Acad. Sci. USA. 78: 6633-37. Other modified nucleotides can be used in analogous ways. That is, other modified nucleotides with minimal modification can be incorporated during replication and labeled after incorporation.

Examples of labels suitable for addition to AAdUTP are radioactive isotopes, fluorescent molecules, phosphorescent molecules, enzymes, antibodies, and ligands. Examples of suitable fluorescent labels include fluorescein isothiocyanate (FITC), 5,6-carboxymethyl fluorescein, Texas red, nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), coumarin, dansyl chloride, rhodamine, amino-methyl coumarin (AMCA), Eosin, Erythrosin, BODIPY®, Cascade Blue®, Oregon Green®, pyrene, lissamine, xanthenes, acridines, oxazines, phycoerythrin, macrocyclic chelates of lanthanide ions such as quantum dye™, fluorescent energy transfer dyes, such as thiazole orange-ethidium heterodimer, and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. Examples of other specific fluorescent labels include 3-Hydroxypyrene 5,8,10-Tri Sulfonic acid, 5-Hydroxy Tryptamine (5-HT), Acid Fuchsin, Alizarin Complexon, Alizarin Red, Allophycocyanin, Aminocoumarin, Anthroyl Stearate, Astrazon Brilliant Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, Auramine, Aurophosphine, Aurophosphine G, BAO 9 (Bisaminophenyloxadiazole), BCECF, Berberine Sulphate, Bisbenzamide, Blancophor FFG Solution, Blancophor SV, Bodipy F1, Brilliant Sulphoflavin FF, Calcien Blue, Calcium Green, Calcofluor RW Solution, Calcofluor White, Calcophor White ABT Solution, Calcophor White Standard Solution, Carbostyryl, Cascade Yellow, Catecholamine, Chinacrine, Coriphosphine O, Coumarin-Phalloidin, CY3.1 8, CY5.1 8, CY7, Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid), Dansa (Diamino Naphtyl Sulphonic Acid), Dansyl NH-CH_3, Diamino Phenyl Oxydiazole (DAO), Dimethylamino-5-Sulphonic acid, Dipyrromethemeboron Difluoride, Diphenyl Brilliant Flavine 7GFF, Dopamine, Erythrosin ITC, Euchrysin, FIF (Formaldehyde Induced Fluorescence), Flazo Orange, Fluo 3, Fluorescamine, Fura-2, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow 5GF, Gloxalic Acid, Granular Blue, Haematoporphyrin, Indo-1, Intrawhite Cf Liquid, Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine

Rhodamine B200 (RD200), Lucifer Yellow CH, Lucifer Yellow VS, Magdala Red, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, MPS (Methyl Green Pyronine Stilbene), Mithramycin, NBD Amine, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant Flavin E8G, Oxadiazole, Pacific Blue, Pararosaniline (Feulgen), Phorwite AR Solution, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, Phthalocyanine, Phycoerythrin R, Polyazaindacene Pontochrome Blue Black, Porphyrin, Primuline, Procion Yellow, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, Quinacrine Mustard, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B Extra, Rhodamine BB, Rhodamine BG, Rhodamine WT, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS (Primuline), SITS (Stilbene Isothiosulphonic acid), Stilbene, Snarf 1, sulpho Rhodamine B Can C, Sulpho Rhodamine G Extra, Tetracycline, Thiazine Red R, Thioflavin S, Thioflavin TCN, Thioflavin 5, Thiolyte, Thiozol Orange, Tinopol CBS, True Blue, Ultralite, Uranine B, Uvitex SFC, Xylene Orange, and XRITC.

Preferred fluorescent labels are fluorescein (5-carboxyfluorescein-N-hydroxysuccinimide ester), rhodamine (5,6-tetramethyl rhodamine), and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. The absorption and emission maxima, respectively, for these fluors are: FITC (490 nm; 520 nm), Cy3 (554 nm; 568 nm), Cy3.5 (581 nm; 588 nm), Cy5 (652 nm: 672 nm), Cy5.5 (682 nm; 703 nm) and Cy7 (755 nm; 778 nm), thus allowing their simultaneous detection. Other examples of fluorescein dyes include 6-carboxyfluorescein (6-FAM), 2',4',1,4,-tetrachlorofluorescein (TET), 2',4',5',7',1,4-hexachlorofluorescein (HEX), 2',7'-dimethoxy-4', 5'-dichloro-6-carboxyrhodamine (JOE), 2'-chloro-5'-fluoro-7',8'-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED), and 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC). Fluorescent labels can be obtained from a variety of commercial sources, including Amersham Pharmacia Biotech, Piscataway, NJ; Molecular Probes, Eugene, OR; and Research Organics, Cleveland, Ohio.

Another useful label, related to molecular beacon technology, is Amplifluors™. Amplifluors™ are fluorescent moieties and quenchers incorporated into primers containing stem structures (usually in hairpin or stem and loop structures) such that the quencher moiety is in proximity with the fluorescent moiety. That is, the quencher and

fluorescent are incorporated into opposite strands of the stem structure. In the structured state, the quencher prevents or limits fluorescence of the fluorescent moiety. When the stem of the primer is disrupted, the quencher and fluorescent moiety are no longer in proximity and the fluorescent moiety produces a fluorescent signal. In the disclosed method, use of Amplifluor™ primers in ERCA produces double stranded tandem sequence DNA where the primer stem is disrupted in favor of a complementary, replicated strand. From a reaction initially containing structured (that is, non-fluorescent) Amplifluor™ primers, fluorescence signal increases as amplification takes place, as more and more of the Amplifluor™ primers are incorporated into double stranded TS-DNA, as the Amplifluor™ stems are disrupted, and as the fluorescent moieties as consequently unquenched. Thus, use of Amplifluors™ is particularly suited for real-time detection of amplification in ERCA.

The amplification products of RCA can be detected using any suitable technique. Real time detection, that is, detection during the RCA reaction is a preferred mode of detection with the disclosed method. Real time detection can be facilitated by use of Amplifluor™ primers. Amplifluor™ primers produce a fluorescent signal when they become incorporated into a replicated strand and are based paired with a complementary strand.

2. Secondary Labeling with Detection Probes

Secondary labeling consists of using suitable molecular probes, referred to as detection probes, to detect the amplified DNA or RNA. For example, an amplification target circle may be designed to contain several repeats of a known arbitrary sequence, referred to as detection tags. A secondary hybridization step can be used to bind detection probes to these detection tags. The detection probes may be labeled as described above with, for example, an enzyme, fluorescent moieties, or radioactive isotopes. By using three detection tags per amplification target circle, and four fluorescent moieties per each detection probe, one may obtain up to twelve fluorescent signals for every amplification target circle repeat in the TS-DNA, yielding up to 12,000 fluorescent moieties for every amplification target circle that is amplified by RCA.

3. Multiplexing and Hybridization Array Detection

RCA is easily multiplexed by using sets of different amplification target circles, each set carrying different address tag sequences designed for binding to unique address probes. Note that although the address tag sequences for each amplification target circle are different, the primer complement portion may remain unchanged, and thus the primer for rolling circle replication can remain the same for all targets. The TS-DNA molecules generated by RCA are of high molecular weight and low complexity; the complexity being the length of the amplification target circle. A given TS-DNA can be captured to a fixed position in a solid support by, for example, including within the spacer region of the amplification target circles a unique address tag sequence for each unique amplification target circle. TS-DNA generated from a given amplification target circle will then contain sequences corresponding to a specific address tag sequence.

4. Combinatorial Multicolor Coding

A preferred form of multiplex detection involves the use of a combination of labels that either fluoresce at different wavelengths or are colored differently. One of the advantages of fluorescence for the detection of hybridization probes is that several targets can be visualized simultaneously in the same sample. Using a combinatorial strategy, many more targets can be discriminated than the number of spectrally resolvable fluorophores. Combinatorial labeling provides the simplest way to label probes in a multiplex fashion since a probe fluor is either completely absent (-) or present in unit amounts (+); image analysis is thus more amenable to automation, and a number of experimental artifacts, such as differential photobleaching of the fluors and the effects of changing excitation source power spectrum, are avoided.

The combinations of labels establish a code for identifying different detection probes and, by extension, different analytes to which those detection probes are associated with. This labeling scheme is referred to as Combinatorial Multicolor Coding (CMC). Such coding is described by Speicher *et al.*, *Nature Genetics* 12:368-375 (1996). Any number of labels, which when combined can be separately detected, can be used for combinatorial multicolor coding. It is preferred that 2, 3, 4, 5, or 6 labels be used in combination. It is most preferred that 6 labels be used. The number of labels used establishes the number of unique label combinations that can be formed

according to the formula 2^N-1, where N is the number of labels. According to this formula, 2 labels forms three label combinations, 3 labels forms seven label combinations, 4 labels forms 15 label combinations, 5 labels form 31 label combinations, and 6 labels forms 63 label combinations.

Speicher *et al.* describes a set of fluors and corresponding optical filters spaced across the spectral interval 350-770 nm that give a high degree of discrimination between all possible fluor pairs. This fluor set, which is preferred for combinatorial multicolor coding, consists of 4'-6-diamidino-2-phenylinodole (DAPI), fluorescein (FITC), and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. Any subset of this preferred set can also be used where fewer combinations are required. The absorption and emission maxima, respectively, for these fluors are: DAPI (350 nm; 456 nm), FITC (490 nm; 520 nm), Cy3 (554 nm; 568 nm), Cy3.5 (581 nm; 588 nm), Cy5 (652 nm; 672 nm), Cy5.5 (682 nm; 703 nm) and Cy7 (755 nm; 778 nm). The excitation and emission spectra, extinction coefficients and quantum yield of these fluors are described by Ernst *et al.*, *Cytometry* 10:3-10 (1989), Mujumdar *et al.*, *Cytometry* 10:11-19 (1989), Yu, *Nucleic Acids Res.* 22:3226-3232 (1994), and Waggoner, *Meth. Enzymology* 246:362-373 (1995). These fluors can all be excited with a 75W Xenon arc.

B. Further Amplification

Secondary DNA strand displacement is another way to amplify TS-DNA. Secondary DNA strand displacement is accomplished by hybridizing secondary DNA strand displacement primers to TS-DNA and allowing a DNA polymerase to synthesize DNA from these primed sites. The product of secondary DNA strand displacement is referred to as secondary tandem sequence DNA or TS-DNA-2. Secondary DNA strand displacement and strand displacement cascade amplification are described in U.S. Patent No. 5,854,033 and WO 97/19193. Strand displacement cascade amplification, also referred to as exponential rolling circle amplification (ERCA) is a preferred form of RCA for use in the disclosed method.

In exponential RCA, a secondary DNA strand displacement primer primes replication of TS-DNA to form a complementary strand referred to as secondary tandem sequence DNA or TS-DNA-2. As a secondary DNA strand displacement primer is elongated, the DNA polymerase will run into the 5' end of the next hybridized secondary DNA strand displacement molecule and will displace its 5' end. In this

fashion a tandem queue of elongating DNA polymerases is formed on the TS-DNA template. As long as the rolling circle reaction continues, new secondary DNA strand displacement primers and new DNA polymerases are added to TS-DNA at the growing end of the rolling circle. A tertiary DNA strand displacement primer strand (which is complementary to the TS-DNA-2 strand and which can be the rolling circle replication primer) can then hybridize to, and prime replication of, TS-DNA-2 to form TS-DNA-3 (which is equivalent to the original TS-DNA). Strand displacement of TS-DNA-3 by the adjacent, growing TS-DNA-3 strands makes TS-DNA-3 available for hybridization with secondary DNA strand displacement primer. This results in another round of replication resulting in TS-DNA-4 (which is equivalent to TS-DNA-2). TS-DNA-4, in turn, becomes a template for DNA replication primed by tertiary DNA strand displacement primer. The cascade continues this manner until the reaction stops or reagents become limiting. The additional forms of tandem sequence DNA beyond secondary tandem sequence DNA are collectively referred to herein as higher order tandem sequence DNA. Higher order tandem sequence DNA encompasses TS-DNA-3, TS-DNA-4, and any other tandem sequence DNA produced from replication of secondary tandem sequence DNA or the products of such replication. In a preferred mode of ERCA, the rolling circle replication primer serves as the tertiary DNA strand displacement primer, thus eliminating the need for a separate primer. Exponential rolling circle amplification is further described in U.S. Patent No. 5,854,033 and WO 97/19193 (where it is referred to as strand displacement cascade amplification).

Illustrations

The disclosed method can be further described by the following illustrations.

One form of the disclosed method involving the use of circle capture probes for the detection of HIV p24 antigen is described below.

Microtiter plates will be pre-coated with mouse anti-HIV p24 antibody. Incubation of sample with HIV p24 with antibody-coated microtiter plates will result in the binding of HIV p24 antigen to antibodies anchored on to the plates. Plate bound HIV p24 antigen will then be recognized by polyclonal anti-HIV p24 goat antibody that has been conjugated with the amplification target circle (the conjugate is the reporter binding molecule) and has been preannealed to a RCA circle. Subsequent to washing, captured amplification target circles will be released (decoupled) during ERCA

amplification using appropriate primers. RCA signals will be detected with either a plate reader or ABI-7700 real time instrument and using Amplifluors™ or molecular beacons.

Another form of the disclosed method involving the use of circle linkers for the detection of antigens is described below.

Microtiter plates will be pre-coated with appropriate capture antibodies, in an arrayed fashion, for analyte detection. Incubation of test samples will result in the binding of specific analyte to antibodies anchored on to the plates. Plate bound analytes will then be recognized by a detector antibody that has been conjugated with the amplification target circle (the conjugate is the reporter binding molecule) via a cleavable linker (that is, a circle linker). Subsequent to washing, antibody-conjugated amplification target circle will be released (decoupled), inside a microtiter plate, by cleaving the linker (for example, by DTT treatment to cleave disulfide linkage). Released amplification target circle will be used for signal amplification by ERCA. The signal detection will be carried out with either a real time assay instrument (ABI 7700 sequence detector) or a plate reader using Amplifluors™ or molecular beacons.

Examples

The disclosed method can be further described, and relevant principles illustrated, through the following examples.

A. Example 1: Coating of Micro Amp polypropylene tubes with antibody.

This example demonstrates that Micro Amp tubes can be coated with antibody as efficiently as microtiter plates. Micro Amp polypropylene tubes (appropriate for use in ABI 7700 sequence detector) and polystyrene ELISA microtiter plates were coated with variable amounts of anti-biotin antibody. For this purpose, 40 μl of desired antibody, in 50 mM carbonate buffer pH 9.6, was incubated overnight at 4°C in these tubes. Subsequent to incubation, any uncoated material was washed with 150 mM phosphate buffer saline, pH 7.2. Subsequent to washing, coated anti-biotin antibody was recognized by 1 μg/ml of biotin coupled horse reddish peroxidase (HRP). Subsequent to washing of unbound proteins, the relative amounts of bound HRP were detected by using OPD assay. After 10-15 min, the assay mixture was transferred from Micro Amp tubes to Costar plates and the absorbance of the assay mixture was

evaluated at 450 nm using a plate reader. As shown in Figure 2, both microtiter plates and Micro Amp tubes showed similar levels of antibody coating.

B. Example 2: Detection of amplification target circles, captured on antibody-coated Micro Amp tubes, by immuno ERCA using ABI 7700 sequence detector instrument.

This example demonstrates amplification and detection of captured amplification target circles. The strategy for this example is shown in Figure 3. In this example, variable amounts of reporter binding molecules were used to assess, in part, the effect of the amount of reporter binding molecules on signal over background. Micro Amp tubes were coated, as described in Example 1, with 30 µl of 10 µg/ml of either anti-biotin antibody or mouse IgG. These antibodies serve as "analytes" in this example. Subsequent to washing, the tubes were blocked using blocking solution (blocker casein in PBS, Pierce Chemicals) and washed again with PBS carrying 0.05% Tween 20. In a separate tube, amplification target circle 1822oc88 was annealed to 3'-biotin labeled circle capture probe in 2xSSC. The amplification target circle/circle capture probe/biotin is a reporter binding molecule. The biotin is the specific binding molecule. Various amounts of circle capture probe annealed circles (freshly diluted in 30 µl PBS) were added to coated Micro Amp tubes and incubated at 37°C for 1 hour. Unbound probe-annealed circles as well as probes were washed away using PBS. Trapped circles were detected by ERCA using TET linked Amplifluor™ as one of the two primers and real time detection of fluorescence in ABI 7700 sequence detector. This allowed real-time detection of amplification products as TET moieties emitted fluorescent signal after synthesis of complementary strands. 30 µl of the ERCA mix contained 5% tetramethyl ammonium oxlate, 400 µM dNTP mix, 1 µM each of the two primers, 8 units of Bst DNA polymerase (New England Biolabs, MA), and 1X modified ThermoPol buffer containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO4$ and 0.1% Triton X-100. ERCA reactions were performed at 60°C. Histographic analysis of the amplification results is shown in Figure 4. The graphs show fluorescence detected over time (the time units are labeled as "cycles" in the graphs although there was no cycling involved). Fluorescent signal is observed in assays containing the anti-biotin antibody "analyte" prior to signal seen in the control assays without analyte. Figure 4 notes this difference in signal appearance as ΔCt. As

can be seen, even when very few reporter binding molecules (360) were used, there was still an easily observable difference in the time of signal appearance.

C. Example 3: Detection of the binding of variable number of amplification target circles using fixed number of amplification target circles in immuno ERCA with Amplifluors™.

This example demonstrates detection binding of a fixed number of amplification target circles to a variable number of circle capture probes. The strategy for this example is shown in Figure 5. Because the number of amplification target circles used were the same, the background caused by the circles was expected to be similar in all of the assays. Anti biotin antibody-coated or mouse IgG-coated Micro Amp tubes were used to incubate various amounts of 3'-biotin labeled circle capture probes, in PBS. Subsequent to the removal of unbound probes, $1x10^6$ 1822oc88 amplification target circles, in 2x SSC, were used for annealing to antibody-bound circle capture probes at 37°C for 1 hour. Subsequent to the washing of excess circles, probe-annealed circles were detected by ERCA as indicated before. Amplification products were detected in real time as TET moieties emitted fluorescent signal after synthesis of complementary strands. Difference in Ct values between anti biotin antibody-coated and mouse IgG-coated tubes at various for various amounts of circle capture probes are plotted in Figure 6. The graph shows the difference in the time of fluorescent signal detection (ΔCt) using different amounts of circle capture probes. As can be seen, there was an easily observable difference in the time of signal appearance at all amounts of circle capture probe. These results also demonstrate that variations in the circle capture probe, bound to capture antibodies, can be successfully detected by immuno-ERCA.

D. Example 4: Detection of IL-8 using immuno-ERCA (ERCA-ELISA).

This example demonstrates use of a form of the disclosed method to detect IL-8. The strategy for this example is shown in Figure 7. Micro Amp tubes were coated with 40 µl of 10 µg/ml anti-IL-8 mouse mAb in 50 mM carbonate buffer, pH 9.6, at 4°C for 12 hrs. Variable concentrations of IL-8 (40 µl) were incubated in these tubes, at 37°C, for 1 hr. Subsequent to the washing of unbound IL-8 molecules, 1 µg/ml of biotinylated anti-IL-8 secondary antibody (40 µl) was incubated at 37°C for 1 hr. Subsequent to washing, the tubes were incubated with 40 µl of 10 ng/ml anti-biotin

antibody that has been covalently conjugated with circle capture probe via its 3' end and is pre-annealed with the 1822in88 amplification target circle at 37°C for 5 hrs. Trapped circles were detected by ERCA using FAM Amplifluors™ as described before. This allowed real-time detection of amplification products as FAM moieties emitted fluorescent signal after synthesis of complementary strands. Differences in ΔCt values between no IL-8 and various concentrations of IL-8, used in this assay, are plotted in Figure 8. The graph shows the difference in the time of fluorescent signal detection (ΔCt) when different amounts of IL-8 were present. As can be seen, the ΔCt increases steadily as the amount of IL-8 increased.

It is understood that the disclosed invention is not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

It must be noted that as used herein and in the appended claims, the singular forms "a ", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a host cell" includes a plurality of such host cells, reference to "the antibody" is a reference to one or more antibodies and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are as described. Publications cited herein and the material for which they are cited are specifically incorporated by reference. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.